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A Systematic Evaluation of Methods to Separate X- and Y- Bearing Sperm

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A systematic evaluation of methods to separate X- and Y- bearing sperm

By

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Abstract

This project was initiated to determine if was possible to enrich either X- or Y- bearing sperm, and hence to preselect the sex of a child. Two of the possible reasons why couple might want to select the sex of a child are firstly because of a family history of an X-linked recessive genetic disorder, which usually only affect sons, and secondly families who have had several children of one sex. For this study, men with three or more children of the same sex were recruited following the publication of an article in *The West Australian* newspaper.

The percentage of X- and Y- bearing sperm within the semen samples of men with three or more children of the same sex was determined using dual colour fluorescence *in situ* hybridisation (FISH). The aim of the investigation was to determine if these men had an altered ratio of X- to Y- bearing sperm, which would explain why these men had a predominance of children of one sex. Comprehensive analyses were also carried out on the semen samples. The reliability of the dual colour FISH technique was established using a number of standard metaphase spreads; from male and female subjects and an individual with Klinefelters syndrome. It was determined that dual colour FISH was a suitable technique for determining the percentage of X- or Y- bearing sperm within a sample.

The semen samples were processed using one of two protocols. Samples from men with three or more daughters were treated using Human serum albumin columns, with the intention of increasing the percentage of Y-bearing sperm within the final fraction. It has been suggested that this method

enriches the Y- bearing sperm from a sample due to the differential motility exhibited by the X- and Y-bearing sperm, although this characteristic has not been proven. Samples from men with three or more sons were processed using 8-layer ISolate® discontinuous gradients, with the aim of enhancing the amount of X- bearing sperm within the final fraction. This method is on based on the studies carried out using Percoll discontinuous gradient. ISolate® was used in the formation of the discontinuous gradients because Percoll has not been approved for the production of sperm fractions for human insemination. It has been suggested that the X- and Y- bearing sperm can be enriched using such gradients either as a result of differences in their velocity of sedimentation or due to a greater nett negative charge on the surface of X- bearing sperm. However, neither of these theories have been validated. As it has also been proposed that the survival rate of X- bearing sperm is slightly longer than that for Y- bearing sperm, this was also investigated.

In summary no statistically significant enrichment of X- or Y- bearing sperm was observed following the treatment of the semen samples with either the ISolate® discontinuous gradient or the Human serum albumin column protocols. Nor was there any enrichment in X- bearing sperm due to their suggested greater survival time.

Declaration

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any institution of higher education; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Signature .....

Date 21/12/98

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List of Abbreviations

α-DIG-RHO	Anti-digoxigenin rhodamine
ACTH	Adrenocorticotropic hormone
ART	Assisted Reproductive Technologies
ASABs	Antisperm Antibodies
ATP	Adenosine triphosphate
AV-FITC	Avidin Fluorescein Conjugated
Biot-αAV	Biotinated Anti-Avidin
BMD	Becker Muscular Dystrophy
BSA	Bovine Serum Albumin
CSD	Complementary Sex Determination
DABCO	1',4'-Diazabicyclo[2.2.2.]Octane
DAPI	4'6' Diamidino-2-phenylindole
DMD	Duchenne Muscular Dystrophy
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EBSS	Earle's Balanced Salt Solution
ESD	Environmental Sex Determination
F-body	Fluorescent body
FA	Formamide
FISH	Fluorescence in situ hybridisation
G-6-PD	Glucose -6- Phosphate Dehydrogenase
GSD	Genetic Sex Determination
HSA	Human Serum Albumin

ISH	*in situ* hybridisation
IVF	*In vitro* fertilisation
KEMH	King Edward Memorial Hospital, Perth, WA
ml	Millilitres
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PGD	Preimplantation Genetic Diagnosis
PMH	Princess Margaret Hospital
SSC	Standard saline concentrate solution
SRY	Sex Determining Region of the Y chromosome
Sxl	Sex Lethal gene
TDF	Testis Determining Factor
TMS	Total Motile Sperm
TSD	Temperature-dependent sex determination
TSP	Temperature-sensitive period
TW20	Tween ® 20
WHO	World Health Organisation

1. Introduction

1.1 Determinants of sex in the animal kingdom

In all mammals, sex is genetically determined at the time of fertilisation, with females usually homogametic, XX and males heterogametic, XY. At fertilisation, the egg always contains an X chromosome, hence the sex is determined by the penetrating sperm. Consequently, fertilisation by an X chromosome-bearing sperm results in a female embryo, while fertilisation by a Y chromosome-bearing sperm leads to a male embryo being conceived.

From studies of individuals with abnormal sex chromosomes, it has been shown that the presence of a Y chromosome is normally the male-determining factor. The mechanisms involved in this determination have been investigated for over forty years. Some of the earlier experiments showed that embryonic testes are required for the development of the accessory male sex organs and the gene responsible for the initiation of testis formation became known as the testis determining factor (TDF). Through the study of individuals with chromosomal abnormalities, the TDF gene was isolated in 1990 to a region on the Y chromosome known as the Sex Determining Region of the Y chromosome or SRY (Sinclair et al., 1990).

In studies involving sex-reversed individuals, examination of XY females indicated that in about 15% of cases the SRY gene was mutated, with 24 mutations being identified in the gene, the majority occurring within the HMG box (Kwok et al., 1996; Veitia et al., 1997).

The observation that SRY is mutated in only approximately 15% of XY females indicates that other genes are almost certainly involved. A second gene that has been suggested in this role is DAX-1, which has been shown to

be duplicated in some XY females. Although the mechanisms by which SRY and DAX-1 cause sex determination remain unclear, it is believed that they may play some role in a complex signalling pathway (Swain, Zanaria, Hacker, Lovell-Badge & Camerino, 1996).

There are a number of different chromosomal abnormalities which can affect the sex of an individual. These variants are generally rare, involving approximately 1 in 500 births, and they are usually phenotypically less severe than the anomalies associated with autosomal chromosome disorders. The four main sex chromosome aneuploidy disorders are Klinefelter syndrome, XYY syndrome, Trisomy X and Turner syndrome. Klinefelter syndrome affects 1 in 1000 male births, with affected males usually having the karyotype 47, XXY. There are also, however, numerous recorded cases of mosaic karyotypes, for example, 46,XY/47,XXY. The frequency of this disorder increases to 100 per 1000 amongst males identified as being azoospermic infertile, and is 10 per 1000 amongst mentally handicapped males. Males with the 47,XXY karyotype are usually tall and thin, and at puberty hypogonadism is observed. In the absence of treatment, the secondary sexual characteristics remain permanently underdeveloped (Connor & Ferguson-Smith, 1997) and Klinefelter males are almost always infertile (Arce & Padrón, 1980).

There is no obvious phenotype associated with 47,XYY syndrome. It was reported that there is a higher proportion of XYY males in Penal institutions for the mentally subnormal, at 20 in 1000 male births, compared with 1 in 1000 male births in the general population. XYY males are slightly taller on average, but have normal body proportions and have an increased risk of behavioural

problems. They also, however, may present with a normal phenotype and unimpaired intelligence and fertility (Connor & Ferguson-Smith, 1997).

Trisomy X (47,XXX) is observed in approximately 1 in 1000 live female births, while tetra- (48,XXXX) and pentasomy X (49,XXXXX) are significantly rarer. No specific abnormal phenotype is associated with Trisomy X, and hence affected females are usually only identified at infertility clinics, if at all. One observation that has emerged from studies of Trisomy X females is that in about 70% of cases the patients have serious learning problems. Tetrasomy and pentasomy X syndrome are more severe, and serious mental and physical problems are often observed with tetrasomy X, while pentasomy X syndrome-affected females frequently exhibit severe developmental retardation and multiple physical defects (Thompson, McInnes & Willard, 1991).

Turner syndrome (45,X) is much less commonly observed than the other syndromes discussed above, affecting only 1 in 5000 live female births. The frequency at conception is significantly higher, but over 99% of affected fetuses are thought to be spontaneously aborted. Thus Turner syndrome is the most common sex chromosome abnormality reported in early spontaneous abortions, with approximately 10% of cases having the karyotype 45,X. Like Klinefelter syndrome in males, mosaics for Turner syndrome are commonly observed. There are a number of physical abnormalities that are typically observed with individuals affected by Turners syndrome, including short stature, characteristic unusual faces, neck webbing and broad chests (Connor & Ferguson-Smith, 1997). An individual with Turner syndrome rarely spontaneously achieves a pregnancy, either because the ovaries fail to fully develop or they undergo degeneration and ultimately disappear (Connor & Ferguson-Smith, 1997).

Women with mosaicism (46,XX/45,X) might be able to use their own oocytes, although in such cases egg donation is becoming more the norm as pregnancies achieved using oocytes from women who are Turners mosaics have elevated levels of chromosome anomalies, fetal malformations, spontaneous abortions and pre-eclampsia (Ditkoff, Vidali & Sauer, 1996; Guerquin, 1993).

Drosophila were initially used as a model for sex determination in higher eukaryotes, although since the studies began it has been demonstrated that there are very few similarities between the underlying mechanism of sex determination in Drosophila and that of mammals. In Drosophila, the primary signal for determining the sex of an embryo is the ratio of the X chromosome to autosomes. In females, the X: A ratio is usually 1.0 because they normally have 2 X-chromosomes and 2 sets of autosomes. On the other hand, males usually only have 1 X chromosome and therefore commonly have a ratio of 0.5. For sex determination, the Drosophila embryo must be able to monitor the number of X-chromosomes. This process is carried out by the Sex lethal gene (Sxl), which is the master switch for sex determination. The twofold difference in the X chromosome gene expression of specific transcription factor encoding genes seen in female embryos is thought to be the method by which Sxl is able to count the number of X chromosomes. When present at twofold levels, it is thought that the transcription factors activate Sxl thus causing it to produce an active form of the Sxl protein and to initiate the pathway for sex determination (Marx, 1995). It is also noteworthy that in Drosophila, sex determination is autonomous; that is, sex determination occurs independently at the single cell level (Parkhurst & Merely, 1994).

Birds, butterflies and moths are included in species whose method of genetic sex determination involves female heterogamety. This means that males are ZZ while females are ZW, the letters Z and W being adopted to prevent confusion with the XY system. As yet, the method of sex determination seen with female heterogamety is not completely understood. In domestic fowl, which are commonly used for such studies, ZZW triploids are typically intersexed. From data derived from these chromosomal variants, it has been suggested that it is the autosome to sex chromosome ratio which is the determining factor for sex, and hence the W chromosome would not be dominant. Confirmation of this hypothesis would be obtained if the ZO aneuploid were female, however this form of chromosomal abnormality has not been observed (Halverson & Dvorak, 1993). The fact that birds, butterflies and moths display female heterogamety does not necessarily mean that the methods involved in sex determination in these species is identical, therefore further studies must be undertaken not only in domestic fowl but in other species displaying female heterogamety.

One mechanism of sex determination that could be considered very different from those already discussed is arrhenotokous reproduction or haplodiploidy. This form of sex determination has been observed in the insect order Hymenoptera, and has been studied in species from both the suborders Symphya and Apocrita. In this reproductive system, males are normally haploid and develop from unfertilised eggs whilst females are diploid and develop from fertilised eggs (Bull, 1979; Cook, 1993). The genetics of arrhenotokous reproduction has been mainly studied in the Apocrita suborder, which is probably due to the unique social features observed in species of bees, wasps

and ants. It has been determined that males only obtain maternal chromosomes, whilst females obtain genetic information from both parents. There are currently two models to explain the mechanisms involved in arrhenotoky; complementary sex determination (CSD) and genetic balance. In CSD, sex is determined by multiple alleles at a single locus; thus females would have to be sex locus heterozygotes while males would be hemizygous. According to genetic balance models, there are both male and female sex-determining factors, and female sex determining factors have to exert a cumulative effect. As yet, the putative mechanisms involved are not fully understood (Cook, 1993).

In a number of reptile and fish species, sex is determined by environmental factors, a form of sex determination which is referred to as Environmental Sex Determination (ESD). This means that the sex of the offspring is determined by environmental cues and it is not due to genetic factors as in Genetic dependent sex determination (GSD). Temperature, pH, and crowding are all factors which influence the ratio of male to female offspring.

Temperature-dependent sex determination (TSD) is the most commonly observed form of ESD, and it is thought to be associated with a lack of heteromorphic sex chromosomes. The exact mechanisms of TSD are not fully understood. Studies have shown that there is a temperature-sensitive period (TSP) during which temperature is known to affect the proportions of male and female offspring. In many of the reptiles investigated, the TSP corresponds to the middle third of the incubation period (Johnston, Barnett & Sharpe, 1995). It has been suggested that the temperature during the TSP acts as some form of biological switch, however the fact that at intermediate temperatures no intersex

offspring are observed indicates that the mechanisms involved must be somewhat more complicated

Examples of TSD can be seen in a number of different animal orders, including Squamata, Chelonia and Crocodylia. Specific examples of TSD include Chelhydra serpentina, or the Common Snapping Turtle. The eggs of this turtle will only develop into males when incubated between 24°C and 27°C; temperatures above or below this critical range result in female offspring (Saunders, 1982).

Not all animals from the Squamata, Chelonia and Crocodylia orders have their sex determined by environmental factors. Within the Squamata order, which contains both snakes and lizards, there are no known examples of snakes using TSD. All species of snakes studied thus far have heteromorphic sex chromosomes, and utilise genetic dependent sex determination. Temperature may however have an influence even in these species; for example, it has been suggested that temperature exerts a differential mortality on eggs, with low temperatures killing male embryos and high temperatures killing females (Deeming & Ferguson, 1988).

A number of animals appear to have the ability to manipulate the sex ratio of their offspring, in order to allow them to exploit changes in habitat conditions. Examples of such manipulation have been observed in mammals, including deer, hamsters and opossums. The altered sex ratios have been noted in a number of studies based on different conditions. For example, in one study when female opossums were fed extra food, they produced more male offspring. Opossums fed extra sardines gave birth to 1.4 males to every female, whereas the sex ratio was equal among the offspring of female opossums not

fed the extra food (Austad & Sunquist cited in Ridley, 1994). This was explained as an effect of evolution observed in polygamous animal societies. In this case, by bearing more male offspring, well-fed female opossums would effectively produce more grandchildren, as their male offspring would in theory be strong because of the extra nutrients during their development and therefore they would have the opportunity to mate with many females. Thus they could father more offspring than any single female could bear. Conversely, if a female was malnourished, any males that were born would be weak and so it would be likely that they would lead a celibate life. Therefore, although female offspring would be likely to produce only a few grandchildren, their contribution to the next generation could still be greater than that of a weak male (Ridley, 1994).

One possible explanation for the above example is that the enhanced diet altered the hormonal levels of the female opossums. It has been demonstrated in a number of studies that diet can influence the levels of hormones in both males and females. For example, a study carried out on young women showed that a diet high in soya beans had a significant effect on both the levels of follicle stimulating hormone and luteinising hormone, although whether such changes could effect the sex of any offspring is as yet unknown (Cassidy, Bingham & Setchell, 1995).

An alternative theory for the observed altered sex ratios is based on the levels of certain hormones, both at the time of conception and during gestation. In humans, it has been suggested that elevated levels of testosterone and oestrogens in either parent at the time of conception increase the probability of sons (James, 1992). However this theory has not been fully investigated. The effect of different hormones on sex-specific embryonic mortality has been

studied in other mammals. In rats, the administration of progesterone and oestrogen at the time of conception respectively produced litters with higher and lower sex ratios (Krackow, 1995). However, the administration of adrenocorticotropic hormone (ACTH) produced female-biased litters in some studies and increased fetal mortality in others. The effect of ACTH is thought to interfere with progesterone secretion and thus lead to an altered reproductive hormone balance (Krackow, 1995).

1.2 Sex ratio in humans

A number of different studies have established that the sex ratio in humans, like that of other mammals is approximately 1:1. On average, at birth the secondary sex ratio ranges between 100 to 110 males per 100 females, which equals a sex ratio of between 0.50 and 0.524 (James, 1987a; Visaria, 1967). However, during the course of the human lifespan, the tertiary sex ratio has a tendency to shift towards an excess of females. One reason for this shift later in life is probably due to females on average having a longer life expectancy (Ruegsegger Veit & Jewelewicz, 1988).

For almost sixty years it has been suggested that there are racial variations in the sex ratio. The first studies were carried out in the United States of America, but since then similar investigations have been completed in various countries where different races co-exist. The results of these studies have shown that the US Black, Chinese and Filipino sex ratios are higher than those of the US White population (James, 1987a). The difference between these sex ratios is usually not highly significant and may not be consistent, for example, a

study by Visaria (1967) showed that the Black sex ratio was 0.507 compared to that for the White population of 0.515. Studies of inter-racial marriages have suggested that the difference between the Black and White sex ratios is largely dependent upon the paternal rather than the maternal ethnicity. An investigation carried out by Khoury, Erickson and James (1984) showed that White fathers had significantly more sons than Black fathers when adjustments had been made for the mother's race. Conversely, White mothers did not have more sons when compared with Black mothers following adjustment for the father's race. However a similar study carried out by Ruder (1986) failed to confirm the findings.

In addition to race, a number of other factors have been suggested to influence the human sex ratio. For example, seasonality (Stolwyk, Aarts, Hamilton, Jongloet & Zielhuis, 1996), android body fat distribution (Singh & Zambarano, 1997), previous induced abortions (Golovachev, Alipov, Novikova & Taptunova, 1980), caloric availability (Williams & Gloster, 1992) and hormone levels (James, 1987b). There are however, a number of limitations with many of these studies and the study populations are usually small and non-randomised. For example, in the study by Singh and Zambarano (1997) on the effect of android body fat distribution on the secondary sex ratio the participants were 69 nurses from a military medical centre. The subject's waist-to-hip ratio (WHR) was measured and used as an indicator of their hormonal profile, because of the suggestion that the WHR is related to the androgenic hormonal profile. It was shown that there was a positive relationship between the women's current WHR and the percentage of sons they had borne. However, this involves the

assumption that a woman's WHR and hormonal profile is stable over their childbearing years.

A common factor in many of the studies is that any observed changes in the sex ratio are very limited. For example, those associated with seasonality, Lyster (1971) noted a difference of 0.0024 in the sex ratio between June and February (James, 1987a).

The distribution of X- and Y- bearing sperm in semen samples is usually approximately in the ratio 1:1 (Wang, Flaherty, Swann & Matthews 1994a). However some studies have indicated that certain individuals consistently have an excess of either Y- or X- bearing sperm in their semen. These studies have generally compared the semen of men who have fathered three or more daughters and no sons, with samples from a control group who have fathered both sons and daughters (Dmowski, Gaynor, Rao, Lawrence & Scommegna, 1979). A study carried out by Bibbins et al (1988) showed that semen samples from men who had fathered three or more daughters and no sons contained 68% X-bearing sperm, with some samples containing up to 79% X-bearing sperm. However the percentage of X- bearing sperm was determined by quinacrine fluorescence staining which, as discussed in chapter 2.6, is now considered to produce unreliable results (van Kooji & van Oost, 1992).

Two hypotheses have been advanced to explain the altered ratio of X- to Y-bearing sperm claimed in these males, the first involving nondisjunction of the Y chromosome at the second meiotic division. An increase in nondisjunction at this stage would result in a decrease in the number of Y- bearing sperm, but it also would cause an increase in the number of sperm containing two Y-

chromosomes. Unusually high levels of sperm with two Y-chromosomes are not however, normally observed (Bibbins, Lipshultz, Ward & Legator, 1988).

The second hypothesis involves the possibility that there is altered composition of the spermatogonial cells. If individuals had two-thirds 46,XY and one-third 46,XX spermatogonial cells, then increased levels of X-bearing sperm such as those observed would be possible. If such a distribution was hereditary, it also would explain why certain families appear to exhibit a bias towards having offspring of the same sex over a number of generations (Bibbins, Lipshultz, Ward & Legator, 1988).

The possibility that men have an altered ratio of X- to Y- bearing sperm is supported by studies which have shown it is not uncommon for families to have multiple children of the same sex. For example, a survey conducted in Australia in 1975 showed that among families with three or more children, over 7 per cent consisted of children of only one sex (English & King, 1983).

1.3 Sex-linked Genetic Diseases

As of January 1998, there are some 8900 recognised single gene disorders, of which just over 500 are X-linked and approximately 27 are Y-linked (Online Mendelian Inheritance in Man, 1998). Three forms of sex-linked inheritance can occur, Y-linked, X-linked dominant and X-linked recessive. Y-linked inheritance, which is sometimes referred to as holandric inheritance, is seen in humans with the transmission of the testis-determining factor (TDF), located on the Y chromosome and only inherited by male offspring. (Connor & Ferguson-Smith, 1997).

X-linked dominant disorders can affect both males and females, however the degree to which the individual is affected is related to their sex. Males are usually uniformly affected with a severe form of the condition, whilst due to X-inactivation females may be variably affected. This variability can arise because within a proportion of cells the X-chromosome carrying the mutant gene will be inactivated, and hence it will not be expressed. When studying the pedigrees of X-linked dominant disorders, considerable similarity can be observed with those of autosomal dominant disorders. The significant difference is however the lack of male to male transmission with X-linked dominant disorders. Examples of X-linked dominant disorders include Vitamin D resistant rickets, and Incontinentia pigmenti. In the disorder Incontinentia pigmenti only affected females are seen, because it has in utero lethality for hemizygous males (Connor & Ferguson-Smith, 1997).

When considering disorders that could be avoided using sex preselection, the most likely candidates are X-linked recessive disorders. There are some 412 X-linked recessive disorders already identified to date in humans. The frequency of these disorders has been shown to vary between ethnic groups. For example, in the United Kingdom the frequency of colour blindness is approximately 80 in 1000 male births, whilst it is very rare in Inuit populations of North America (Connor & Ferguson-Smith, 1997). X-linked recessive disorders have characteristic pedigrees and specific features which include:

a) A preponderance of males are affected.

b) The disease is transmitted by carrier females who are usually asymptomatic; half of the sons of a carrier would be affected and half the daughters would be carriers.

c) If an affected male has children, none of his sons are affected but all of his daughters would be carriers.

d) Affected females can be born if the father is affected and the mother is a carrier.

e) Affected males have unaffected maternal uncles.

Due to X-inactivation, female carriers of X-linked recessive disorders usually are clinically normal but they may have some cells where the normal X chromosome has been inactivated resulting in disease symptoms being exhibited (Davies & Read, 1992). There also is the possibility that a female carrier has a second mutation at the same locus on her other X-chromosome, or in communities where consanguineous marriage is common she has inherited an identical mutation from both parents. In this case the female would show the same clinical features as an affected male.

Duchenne muscular dystrophy (DMD) is one of the more common X-linked recessive disorders, with 1 in 3000 male births being affected. This form of muscular dystrophy is associated with null mutations resulting from frameshifts or nonsense point mutations in the gene for the protein dystrophin, thus preventing its production. The onset of DMD usually occurs by 5 years of age with the progressive weakening of proximal muscles. By the age of 12, most affected males are wheelchair-bound with death usually occurring by 20 years. Prenatal diagnosis of DMD is possible by DNA analysis of chorionic villus samples (Connor & Ferguson-Smith, 1997).

Becker muscular dystrophy (BMD) is the second main form of muscular dystrophy, with onset in late childhood. Symptoms include progressive muscular weakness, calf pseudohypertrophy, marked elevation of serum

creatine kinase and a reduction in levels of dystrophin. Individuals affected with BMD are usually chair-bound approximately 25 years after onset, but do have a severely reduced lifespan. In BMD, the mutations within the dystrophin gene result in reduced levels of functional dystrophin or dystrophin with aberrant function. Like DMD, prenatal diagnosis by DNA analysis of chorionic villus samples is currently available (Connor & Ferguson-Smith, 1997).

Another important example of X-linked recessive inheritance is the erythrocyte enzyme disorder, glucose-6-phosphate dehydrogenase (G6PD) deficiency. Unlike DMD, this disorder does not usually cause chronic ill-health, although acute haemolytic episodes can be induced by a variety of environmental factors, such as consuming either aspirin or broad beans. Female carriers are normally asymptomatic but may be identified following prolonged neonatal jaundice or haemolytic episodes following environmental triggers. G6PD deficiency may be common, with up to 65% of Saudis, 32% of Greeks and 20% of Italians being affected world-wide (Connor & Ferguson-Smith, 1997).

Table 1.1 illustrates the numbers of DMD, BMD and G6PD deficiency cases born in Western Australia between 1990 and 1996. The total births during the period were 178,309.

Table 1.1: Incidence of X-linked recessive disorders in Western Australia between 1990 and 1996

X- linked recessive disorder	Number of live births	Percentage of total number of live births	Number of terminations
DMD	4	0.0020	5
BMD	1	0.0005	0
G6PD deficiency	31	0.0170	0

As can be seen in Table 1.1, although certain sex-linked recessive diseases can cause chronic ill health and even lead to premature death, there are very few births of affected children. It is however possible that the number of affected persons may prove to be higher where the disorder is not diagnosed until later in life (Birth Defects Registry of Western Australia, 1997).

2. Attempts to alter the human sex ratio

2.1 Abortion and Infanticide

Infanticide could be considered to be the oldest form of sex selection, or more accurately de-selection. Japanese birth records from 1600 to 1868 show a ratio of 9 males to 1 female birth being registered. While this in itself does not prove the use of infanticide in Japan during this period, it does strongly suggest that some form of sex selection was occurring (Kerin, 1986). Although now universally condemned worldwide infanticide still occurs, especially in countries where one sex is favoured over the other. For example, there is a strong preference for male children in India, where sons are held in high regard because they are regarded as an asset to the family's labour force. Male offspring are also expected to look after their parents in their old age and it is only sons who can perform certain Hindu funeral rites. Daughters on the other hand move into their husband's home after marriage and so cannot be expected to provide support to their parents. In addition, the parents of daughters are expected to pay a dowry at the time of her marriage, and subsequently perform a number of ceremonies annually, for which they are financially responsible. These factors have led some communities to adopt infanticide in order to ensure that they have only or mainly male children (Anand-Kumar, 1995). The most traditional methods of infanticide include putting opium on the mother's nipple before feeding the baby, placing the placenta over an infant's face, or feeding the child with milk from poisonous oleander berries (Patel, 1989; Sermon, Lissens, Joris, Van Steirtegham & Liebaers 1996). There have been a number of studies carried out in India into the prevalence of female infanticide. Some studies have identified different distributions in female infanticide use, for

example Visaria (1994) stated that there was a distinct difference between North and South India, due to the higher status of women in the southern states of India. Other studies have stated that female infanticide is greater in the poorer states, such as Tamil Nadu and Bihar (De Lamo, 1997). In general terms, female infanticide appears to occur throughout India although at variable levels.

With improvements in medical technology, at least in urban areas there seems to be a move away from the manipulation of the secondary sex ratio by infanticide towards sex determination followed by sex-selective abortion. The sex determination is carried out using sonography, fetoscopy, chorion villus biopsy or amniocentesis. In India, amniocentesis and ultrasound have been the most popular methods of determining the sex of a fetus. Amniocentesis was introduced in 1952 and became widely available by the mid-1970s (Stranc, Evans & Hamerton 1997). The process involves the pricking of the fetal membrane with an ultrasound-guided needle and removing approximately 20 ml of amniotic fluid. Fetal cells are separated from the amniotic fluid for chromosomal analysis. The procedure was originally developed to detect single gene and chromosomal disorders and neural tube defects. Although chromosomal analyses are usually between 99.4% and 99.8% accurate, in approximately 1% of cases there may be the risk of spontaneous abortion, premature delivery, dislocation of the hip, respiratory complications or needle puncture marks on the baby (Stranc, Evans & Hamerton, 1997).

Diagnostic ultrasound (DU) was introduced for medical purposes in 1942 by the Austrian Karl Th. Dussik (Levi, 1997), it was however not until after the Second World War that the technique was fully investigated. Ultrasound was being used worldwide for a number of different diagnostic procedures in the field

of gynaecology by 1975. Since the initial form of ultrasound was developed, the technique has evolved due to improvements in technology and today ultrasound is widely used in numerous clinical situations (Levi, 1997). Since the early 1980s it has been suggested that it would be possible to determine the sex of a fetus in the early part of the second trimester using ultrasound. Previously the image resolution of the technology only allowed genitalia visualization in the later stages of gestation. An example of such a study was that by Plattner et al (1983), involving 367 pregnancies, with the sex of the fetus correctly indicated in 93% of cases. More recently, the sex of the fetus was correctly identified, between 9 and 21 weeks gestation, in 99.3% and 96.7% of cases (Harrington, Armstrong, Freeman, Aquilina & Campbell, 1996; Meagher & Davison, 1996). The level of success does appear to be variable and may be dependent on the skill of the operator; therefore it is possible that the error rate would be higher with a less experienced operator.

In many countries, amniocentesis is subject to strict controls and it is relatively expensive, but in India the test is widely available and costs approximately US$6 to US$40, with the selective abortion costing a similar amount (Patel, 1989). For some families it is considerable preferable to spend up to US$80 for sex determination testing and possible selective abortion than to have a female baby and subsequently have to spend thousands of rupees for her marriage (Patel, 1989).

There have been several estimates as to the number of female fetuses aborted after sex determination tests. One such estimate from a Times of India editorial in June 1983 was that between 1978 and 1983 approximately 78000 female fetuses were aborted after sex determination (cited by Patel, 1989).

Another suggestion from government reports is that approximately 50,000 female fetuses are aborted annually after sex determination tests (cited by Iman, 1994). In response to these levels of selective abortion of female fetuses the Indian Houses of Parliament passed the Prenatal Diagnostic Techniques Bill in 1994. The Bill represents an addition to articles 14 and 15 of the Indian constitution that forbids discrimination against women, and hence prohibits abortions due solely to the sex of the fetus. Under the new Bill, medical practitioners identified as offering to determine the sex of a fetus will be struck off the medical register and will face up to three years in prison and a fine of up to 10000 rupees. Pregnant women who undergo the test will be subject to a similar fine and prison term. There have been concerns expressed about the enforceability of this law, due to possible misuse of legal prenatal diagnostic techniques and the fact that many women are forced into having such tests by relatives. The new Bill does not completely ban the use of prenatal diagnostic techniques, where there is a history of genetic disorders within the women's family, and it has been suggested that families might provide false medical histories in order to obtain the tests. Another major concern is that the removal of selective abortion as an option will result in a resurgence in the levels of infanticide (Booth, Verma & Beri, 1994; Imam, 1994).

Sex-selective abortion and infanticide are both based on the theory that the smaller the victim the lesser the crime. Abortion is a controversial subject in itself, but its use to selectively eliminate fetuses simply because of their sex could be considered feticide. Many doctors agree that sex selection by selective abortion should only be used when there is no alternative, and where there is a strong indication that the fetus is affected with a specific sex-linked disorder.

In Western Australia, abortion is illegal under sections 199, 200 and 201 of the West Australian Criminal Code. These sections are based on the British Offences Against the Person Act 1861, sections 58 and 59 and:

"prohibit unlawful administration of any poison or noxious thing, or use of any instrument or other means, with intent to procure miscarriage. The crime of unlawful abortion may be committed by the women herself, by the abortion, or by anyone who supplies or procures anything to aid the abortion" (Cica, 1991).

However there has been no recent enforcement of these sections, and therefore within metropolitan Perth abortion is almost available on demand. It has been suggested that this state of non-enforcement has arisen because of the interpretation that an abortion is not unlawful when it is "performed in good faith for the preservation of the woman's physical or mental health" (Cica, 1991).

2.2 Preimplantation Genetic Diagnosis

Preimplantation genetic diagnosis (PGD) of human embryos was first reported over several years ago, and up to September 1997 over 40 babies had been born worldwide using the technique (Grofi, Tang & Krey, 1997).

There are three methods that can be used to biopsy an embryo;

1. The separation of cells at the two-cell stage.
2. The removal of one or two cells at the four to eight cells stage.
3. The removal of part of the trophectoderm from the blastocyst.

Genetic analysis of the removed cells to determine the sex of the embryo can also be performed in a number of ways, for example:

1. Y- chromosome specific DNA probes.
2. Measurement of dosage differences in metabolic activity of gene products of the X-chromosome prior to inactivation.
3. DNA amplification of a Y- specific sequence using polymerase chain reaction (PCR).
4. Fluorescence *in situ* hybridisation using Y- specific probes.

(Royal Commission on New Reproductive Technologies, 1993a)

To reduce the risk of misdiagnosis, some clinics use X- and Y- specific sequence probes. Not only should this permit unequivocal diagnosis of the sex of the embryo, but also provide information on chromosome copy numbers, thus avoiding the transfer of embryos with abnormal numbers of sex chromosomes (Delhanty, 1994). The basis of the technique is that at the stage when cells are removed the genetic content of all the cells of the embryo are identical, and because the cells are removed before differentiation begins the remaining cells have the potential to develop into a normal embryo. There is however, some debate about this rationale, studies using donated embryos have shown that not all the cells within an embryo are identical. For example Haper and Delhanty (1996) found that:

> In the majority of cases the embryos were diploid mosaics, whereby most cells were diploid, with between one and three cells being aneuploid. In a few cases, especially when examined for autosomes, every nucleus within an embryo was abnormal.

Studies on the viability of biopsied pre-embryos show no detrimental effects of PGD, and this has to some degree been confirmed by the birth of over 40 healthy babies. It should however be noted that a sample of 40 babies is not enough to prove that PGD is completely safe (Jones, 1994; Verlinsky & Kuliev, 1994; Harper, 1996).

Some of those involved in the use of this technique consider it as experimental. Lord Winston, one of the pioneers of the technique at the Hammersmith Hospital, London, said that PGD "was still an experimental procedure that could result in damage to the embryo and should not be used except where there was no alternative" (Fletch, 1997). Due to the concerns surrounding the technique, in the U.K. it can only "be used to overcome the inheritance of sex-linked diseases and not for social reasons" (Fletch, 1997). This however has not stopped a British doctor from setting up a clinic in Italy, where regulations governing PGD are less stringent, to carry out preimplantation sex selection for social reasons (Fletch, 1997).

The regulation of PGD within Australia varies by State. In Western Australia, the Human Reproductive Technology Act, 1991 states that diagnostic procedures cannot be carried out upon an egg in the process of fertilization, or on any embryos, without specific approval by the Western Australian Reproductive Technology Council. The Act also states that the Council will not grant approval for any diagnostic test unless the Council is satisfied that the proposed procedure is intended to be therapeutic for that egg or embryo, and that existing scientific and medical knowledge indicate that no detrimental effects on the well-being of the egg or embryo is thereby likely to occur.

In theory, this means that PGD for the prevention of serious sex-linked diseases could be carried out in Western Australia, but it is as yet not available. At present, the nearest facility offering PGD is in South Australia. In those states allowing PGD, it is only used if there is significant evidence for serious sex-linked diseases, for example, Duchenne's muscular dystrophy, haemophilia and X-linked mental retardation syndrome.

In two reports from the Royal Commission on New Reproductive Technologies in Canada the practical and ethical considerations of the use of PGD were considered (Royal Commission on New Reproductive Technologies, 1993a and b). A number of potential benefits and problems which could result from PGD were suggested, even when solely considering its use for the prevention of serious sex-linked diseases. The main benefit to couples primarily stemmed from the fact that they would be in theory "spared the trauma of undergoing or at least having to consider a therapeutic abortion" (Royal Commission on New Reproductive Technologies, 1993b).

There are two main risks associated with PGD, the first is due to IVF being an essential part of the procedure. IVF is known to involve two main risk areas, those due to the medications that are required and those associated with necessary surgery. Oocyte collection can involve laparoscopy although Transvaginal ultrasound is becoming the preferred method of visualizing the ovaries during oocyte collection. Laparoscopy requires a general anaesthesia, which involves the risk of allergic rashes, temporary paralysis, vomiting and in extreme cases death. The laparoscopy can result in accidental bowel injury, superficial haemorrhage, retained gas, bleeding from the ovary or adjacent

structures and pelvic infection. All of these problems from surgery are rare, especially in fit and healthy women.

Medications are commonly used in the IVF procedure, while the drugs used vary between clinics, all drugs can produce some side effects, such as allergic reactions. The medications used are often associated with stimulating the ovaries to produce more oocytes, for example gonadotropins, the use of these drugs can however, lead to Ovarian hyperstimulation syndrome (OHSS). OHSS is thought to occur in up to 2% of women who under ovarian stimulation and can result in large amounts of fluid accumulating in the abdominal and lung cavities, this can result in kidney damage and thrombosis. The condition whilst very rare can result in death and therefore requires rapid hospitalization and treatment (Concept Leaflet, n.d.; Brinsden, Wada, Tan, Balen & Jacobs, 1995 & Dourron & Williams, 1996).

The second risk associated with PGD is the potential for misdiagnosis, either by not transferring a female embryo because it is thought to be male or by transferring a male embryo because it is thought to be female. Such misdiagnosis has been reported. For example in the report on the procedure in use at the Hammersmith Hospital, of the five pregnancies that had been achieved to 1991 one had been terminated because of misdiagnosis (Royal Commission on New Reproductive Technologies, 1993b). The possibility that embryos are mosaics (Harper & Delhanty, 1996) increases the risk of misdiagnosis and helps to explain the examples of this already cited in the literature.

At present, the technologies associated with PGD have not been developed to levels that make screening for specific genes for all sex-linked

diseases possible. With further developments it should be possible for embryos to be screened for the specific genes, thus allowing the implantation of only healthy male embryos and non-carrier females.

In conclusion, the Royal Commission on New Reproductive Technologies states that PGD is at present "a difficult, expensive and inefficient means of diagnosing genetic disorders prenatally" (Royal Commission on New Reproductive Technologies, 1993b). They did state that further research seems to be justified and that PGD should only be used in a small number of women at high risk of having children with a genetic disorder. This group would not include older women because, although they are at increased risk of having a child affected by chromosomal abnormalities, the level of risk would not be sufficiently high to justify the potential problems and expense currently associated with PGD.

2.3 Diet

An assumption that was popularized in the early 20th century was that sons would be conceived if the mother ate bitter and sour foods and red meat, whilst consuming sweet foods and vegetables would result in the conception of daughters (Gledhill, 1988a). More recently, animal studies have suggested that the relative concentration of sodium, magnesium, potassium and calcium affects the sex birth ratio. One such study was performed on cattle in France between 1967 and 1969 (Stolkowski & Choukroun 1981) and it was demonstrated that a balanced diet would result in equal numbers of male and female offspring.

However, when the diet was rich in alkaline earth metals, such as sodium and potassium, the proportion of female offspring was higher. A similar study using frogs was also carried out, which demonstrated that electrolyte concentrations affect the male to female ratio of developing tadpoles. By comparison, tadpoles raised in a balanced solution of electrolytes resulted in a 1:1 ratio in the adult frogs. Tadpoles raised in a solution that was high in potassium resulted in approximately 70% males, whereas rates of about 70% females were observed using solutions that were high in calcium or magnesium (Rueggsegger Veit & Jewelewicz, 1988).

Stolkowski and Choukroun (1981) applied this theory to humans. They proposed that a diet rich in sodium and potassium and low in calcium and magnesium would result in mainly male offspring, while the opposite would favour female offspring. The suggested diet for male selection consisted of sausage meat, potatoes, beans, artichokes, peaches, apricots and bananas, with the exclusion of dairy products, eggs and greens, and the reverse was true for female selection. The diet was to be followed from the beginning of the menstrual cycle until pregnancy was confirmed (Stolkowski & Choukroun, 1981; Carson, 1988; Rueggsegger Veit & Jewelewicz, 1988). The results reported were that 84% of the 47 couples in the study conceived the child of their stated preference. Although other studies have since been completed, all have involved small study groups and none have included a control group consisting of couples not following the prescribed diet (Stolkowski & Choukroun, 1981; Ruegsegger Veit & Jewelewicz, 1988).

The basic rationale behind this method is that diet influences the ionic milieu of the reproductive tract and ovaries. Thus a sodium- and potassium-rich

diet would serve to produce a more alkaline cervical mucus which would favour Y- bearing sperm. There were however, no direct assays of electrolyte concentrations to determine if such a change had been effected (Kerin, 1986; Carson, 1988). From *in vitro* studies, it has been seen that ionic factors, specifically pH, influence the fertilization of the oocyte. However, as yet it is not known whether the pH of the reproductive tract and ovaries does affect fertilization *in vivo* (Batzofin, 1987).

A number of other studies have been reported, which seem to contradict the experimental basis of the theories proposed by Stolkowski and co-workers. Two studies carried out using animal models did not support the theory that diets high in sodium and potassium but low in calcium and magnesium favour male offspring. The first study involved sows fed diets with varying levels of sodium, magnesium, potassium and calcium, and the overall results showed that diet did not have a significant and reproducible effect on the sex ratio of the offspring (Bolet, Gueguen, Dando & Olliver, 1982). In the second study, female rats were fed varying amounts of sodium chloride, thus altering the sodium to calcium and magnesium ratio. This showed that, with increasing levels of sodium chloride in the diet, there was a decreasing proportion of males within the litters, i.e., a finding contrary to what would have been expected from the original theory associated with sex selection by diet (Bird & Contreras, 1986).

An example of sex selection that is thought to be dependent on dietary intake has been reported from Japan and involves a medication known as Mical. The principal component in Mical is calcium, however the exact concentration of calcium and the overall composition of the preparation has not been disclosed. Mical is prescribed when parents would prefer a son although, according to the

Stolkowski theory, Mical should increase the chance of having a daughter by reducing the sodium and potassium to calcium and magnesium ratio (Batzofin, 1987).

2.4 The timing of coitus

Some of the first reports of sex preselection in humans this century involved the timing of coitus in relation to ovulation. The as yet unproven theory behind this form of sex preselection is that Y- bearing sperm move faster but do not survive as long as X- bearing sperm. In effect, intercourse before or remote from ovulation is more likely to result in female offspring, while intercourse during or close to ovulation should increase the chance of male offspring. A number of different methods are commonly used to determine the timing of ovulation, including measurement of the thermal shift in the basal body temperature, monitoring for changes in volume or consistency of cervical mucus and determining the leutinising hormone peak (Kerin, 1986; Reubinoff & Schenker, 1996).

This theory of sex preselection formed the basis of the "Shettles Method", which has been published in various articles and books in several languages (Shettles & Rorvik, 1970). Shettles developed the method after claiming to have identified two different sperm populations under phase contrast microscopy. One population contained sperm that had small rounded heads, which he identified as Y- bearing sperm. The second population was characterized as having larger oval-shaped heads, and they were believed to be X-bearing.

Other researchers have widely and severely criticized the proposed method and it has been generally described as an optical aberration associated with phase contrast microscopy (Diasio & Glass, 1971). Despite the criticism, Shettles hypothesized that under ideal conditions within the female reproductive tract the smaller Y- bearing sperm would be able to migrate faster and therefore reach the oocyte first. Shettles interpreted these optimal conditions for the ascent of sperm in terms of the conditions of the reproductive tract at the time of ovulation. The cervical mucus has maximal alkalinity at ovulation, however studies of sperm migration through cervical mucus have shown that migration is not in fact influenced by the pH of the mucus (Diasio & Glass, 1971). A criticism of the main study carried out by Diasio and Glass is that their method of determining the percentage of Y- and X- bearing sperm in samples relied on quinacrine dihydrochloride staining of the Y- chromosome, and as noted earlier and in chapter 1.5 more resent studies have shown that the method also stains autosomes and, as such, must be considered unreliable (van Kooji & van Oost, 1992).

The complete protocol developed by Shettles comprises a number of factors:

Table 2.1: Shettles method of sex preselection

Selection for Males	Selection for Females
Mildly alkaline douche	Mildly acidic douche
Intercourse occuring on expected day of ovulation	Intercourse occuring no closer than two or three days before expected ovulation date
Deep penetration	Shallow penetration
Female orgasm before or simultaneous with male	No female orgasm

Shettles has stated that from his communications with over three thousand individuals who used his technique, 84% of those using male preselection and 82% of those using female preselection had conceived the child of their choice (Zarutskie, Muller, Magone & Soules, 1989). There are however some concerns about these claims, as rigorous follow-up was not possible. Some degree of ascertainment bias might apply, and it has been suggested that couples whose expectations have been met would be more likely to report than those who were unsuccessful (Kerin, 1986; Ruegsegger Veit & Jewelewicz, 1988).

Other more controlled studies using the Shettles method have displayed varying levels of success. For example, Simcock (1985) reported no increase in the chances of conceiving a child of the desired sex using the technique (see also Gray, 1991). One must also consider what this method of sex preselection requires of couples, and whether couples can truly follow the instructions and verify the actual sequence of events. Large-scale, controlled studies are required before the effect of the Shettles Method on the secondary sex ratio can be determined with confidence (Carson, 1988).

Studies by Spira *et al* (1988) based solely on the timing of coitus have shown no changes in the secondary sex ratio. The studies included prospective surveys of over 2,300 couples, retrospective studies of more than 5,000 women and results collected from 1,100 women inseminated with donor sperm (Carson, 1988). The findings thus further confuse the issue on the success on coital timing for sex preselection, and before the Shettles method could be accepted as reliable, additional surveys would be needed with sample sizes similar to

those used by Spira and involving accurate methods of determining the timing of ovulation.

2.5 Sperm Separation

Over the past three decades, numerous reports have been published which claim to demonstrate the use of various physical methods to enrich X- or Y- bearing sperm. Many of these studies remain unestablished because the various investigators use modifications of the original method or the results cannot be widely reproduced. The physical differences used to separate these two sperm populations include shape, size, mass, density, electric charge and the presence of H-Y antigen. Differences in each of these factors are likely to be small and hence would require very sensitive techniques. This problem was considered by Beatty (1970) who stated:

> "nature having gone to all the trouble of arranging for a certain proportionality between sexes is unlikely to have given different phenotypes to X- and Y- bearing spermatozoa, with the danger that fortuitous environmental fluctuations might affect the one kind of spermatozoa rather than the other and thus give uncontrolled fluctuations in the ratio of the sexes."

Some of the more promising techniques to affect the enrichment of X- and Y- bearing sperm include flow cytometry, laminar flow, Sephadex gel filtration, swim-up procedures, albumin gradients and Percoll gradients. Flow

cytometry enrichment is based on the difference in the DNA content of X- and Y-bearing sperm, and was initially developed for selective animal breeding. In humans, there is thought to be approximately 3 percent more DNA in an X-bearing sperm than Y- bearing sperm, and because of this difference Y-bearing sperm may be smaller and lighter then their X- bearing counterparts. In other mammals the difference between X- and Y- bearing sperm is more pronounced with, for example, up to a 12.5% difference being observed in the Creeping vole (Johnson, 1995). Flow cytometry involves the use of sophisticated equipment and DNA binding agents, and to sort X- and Y- bearing sperm it requires a number of specific modifications (Johnson & Pinkel, 1986).

Initially the sperm are stained using a fluorescence DNA-binding dye such as Hoechst 33342, and after flow cytometry treatment they are incubated in egg yolk medium to increase the proportion of live sperm. A number of different types of flow cytometers have been used, however the basic principle is the same in all cases. The plane of the sperm nuclei is orientated so that they are all in the same direction, they are then illuminated by a laser beam and the fluorescence emitted is detected by two photomultiplier tube detectors positioned at 0° and 90°. The use of laser light and DNA binding agents, two known mutagenic agents have led to a number of people expressing concern about the safety of the method. For example, Ashwood-Smith (1994) said, "...I would advise caution before the interesting method of separation [flow cytometry] ... is applied to gender selection in fertility centres.". Even though these concerns have been expressed, flow cytometry is still being used in both animals and humans, on the grounds that studies of flow cytometry have successfully produced over 300 offspring from a number of animal species.

Studies on the success of flow cytometry have indicated that it is possible to sort populations of sperm to produce final fractions containing up to approximately 80% of the desired sperm (Johnson, Welch, Keyvanfar, Dorfmann, Fugger & Schulamn, 1993). The Genetics and IVF Institute from Virginia, USA (undated), also advertise flow cytometry for human use on the Internet.

Laminar flow was initially developed to remove dead sperm and debris from semen samples. The underlying theory is that when flow velocity is applied, all live sperm swim in an almost straight pattern. X-bearing sperm swim in a straighter pattern than Y- bearing when a gradient of velocity is introduced, thus allowing possible isolation of the two types of sperm. Using DNA probes, up to 80% enrichment for both X- and Y- bearing sperm has been claimed. The method has not been independently confirmed and so further studies including breeding programmes are required (Gledhill, 1988a).

Sephadex gel is one of the many materials that have been used to produce filtration columns, for sperm enrichment the principle being that larger molecules are separated from smaller ones due to the entrapment of smaller molecules within the gel matrix. Studies such as that by Steeno et al (1975) claimed an enrichment of X-bearing sperm of between 69% and 78%, because X-bearing sperm are larger in size than Y- bearing sperm. However, it should be noted that the proportions of X- and Y- bearing sperm following enrichment was determined using Quinacrine staining, which as stated before is unreliable (also see chapter 2.6). Breeding trials of Sephadex-separated sperm were carried out by Henriet and Jaumotte (1979), who found that cows inseminated with sperm after Sephadex treatment did not produce offspring with an altered sex ratio. A similar study carried out using a human test population was carried

out by Adimoelja (1987). Although this study reported that of the women inseminated with sperm after Sephadex treatment, 27% had daughters. The report, however, fails to state the sex of the children born to other 73% of women who were inseminated with the treated sperm but were then lost from the study. It is very difficult to state that the study was a complete success when there is the possibility that all of the 73% of the women went on to bear sons (Gledhill, 1988b).

It was suggested by Eichwald and Silmser (1955, cited in Bennett & Boyse, 1973) that the surface of Y- bearing sperm would have Y- specific antigens, known as a Y- linked histocompatibility (H-Y) antigen, which led to the theory that it would be possible to develop an anti-H-Y antigen to inactivate all Y- bearing sperm within a semen sample. The theory was tested in several studies using mice. In a study carried out by Goldberg and colleagues (1971), sperm were treated with antisera to the H-Y antigen before being used to inseminate females. Litters from females inseminated with treated sperm contained 45.5% males compared with 53.4% males in the control group. Although this suggested partial success with some reduction in the proportion of males, antisera treatment destroyed between 70% to 80% of the sperm within the samples. This severe reduction in the numbers of live sperm within a sample means that the technique has to be considered unsuitable for clinical use.

Swim-up procedures are used to obtain fractions of motile sperm. There are a number of different possible protocols by which "swim-up" is carried out, for example the incubation period is often varied. Using results obtained from quinacrine staining studies and pregnancies following insemination, it was

suggested by Check *et al* (1989) and Check and Katsoff (1993) that the percentage of Y- bearing sperm could be enriched to over 80% using a modified swim-up protocol.

More recent studies carried out using dual-colour FISH (Han *et al*, 1993) and PCR (Lobel *et al*, 1993) failed to observe any significant enrichment of either X- or Y- bearing sperm following sperm preparation by a swim-up protocol. However, because of the differences in swim-up protocols, more controlled studies were suggested as being necessary. One such study recently completed by De Jonge and Flaherty (Flaherty & Matthews, 1996), failed to produce any enrichment of clinically significant value.

Techniques of this nature are examples of the increasingly sophisticated technology that is considered the most promising form of sex selection. At present, the prevailing opinion appears to be that further studies are required for all of the procedures that have been suggested. The ideal success rate of at least 90% has not as yet been achieved, nor is there evidence of a widely reproducible technique. In fact this is the same conclusion as Beatty had reached in 1974:

> " There does not seem to be at present any technique giving a substantial degree of control of the sex ratio easily repeatable in numerous laboratories. 'Statistically significant' positive findings are published but are offset to some extent by failures to confirm. Further, it can be assumed that all positive findings are published, but not all negative ones, thus introducing a bias into the world of literature."

2.6 Determination of Sex Ratio

The success of methods to alter the sex ratio is measured either by the secondary sex ratio or using laboratory-based analysis. Such laboratory-based forms of analyzing the sperm sex ratio include pseudo-fertilization of hamster eggs, polymerase chain reaction (PCR) studies, and quinacrine fluorescent staining.

Pseudo-fertilization of hamster eggs allows the detection of both X- and Y- bearing sperm. A number of attempts were made to reactivate the sperm nucleus by fusing sperm cells with cultured somatic cells, however in all of these studies the nuclei were not reactivated. When sperm are fused with an oocyte, the tightly packed sperm chromatin is decondensed thus allowing direct analysis. The use of hamster oocytes to reactivate sperm DNA was first suggested by Yanagimachi and colleagues (1976) as a means of assessing sperm for their ability to penetrate zona-free oocytes. The technique is expensive and time-consuming, largely due to the number of oocytes required to test each semen sample. For example, Rudak and colleagues (1978) estimated that approximately 40 to 50 oocytes are recovered from each superovulated female hamster, however approximately 200 oocytes are required per semen sample. Of the approximately 200 oocytes that are used, usually only up to 75 are suitable for chromosomal analysis. Thus, because of the cost of obtaining hamster oocytes, the method is impracticable for analyzing large numbers of sperm from any one individual as would be required for well-controlled studies of sperm enrichment techniques (Goldman et al., 1993). One possibility that has been suggested but not investigated is that because the process is dependent

upon the biological function of the sperm, it is possible that the genetic content of the sperm influences its fertilizing ability and therefore either X- or Y- bearing sperm might have an increased likelihood of fertilizing oocytes (Holmes & Martin, 1993). In any event, this technique cannot be used in Australia, where female hamsters and hence their oocytes are unobtainable due to an importation ban. Although zona-free eggs from other species have been studied, hamster eggs appear to be unique because they can be penetrated by sperm from unrelated species (Yanagimachi, 1982).

An alternative method that has been suggested involves the use of cell-free egg extracts. Gordon et al (1985) have demonstrated that the incubation of human sperm in amphibian egg extract causes chromatin decondensation and chromosome formation. Other studies have shown that egg extracts also stimulate DNA synthesis and the formation of pronuclei and condensed chromosomes in human sperm (Brown, Blake, Wolgemuth, Gordon & Ruddle, 1987; Ohsumi, Katagiri & Yanagimachi, 1988).

Mullis developed PCR in 1983, for which he received the Nobel Prize for chemistry in 1993. PCR has revolutionized biology and medicine and is now regarded as an indispensable method for use in fields such as disease diagnosis, forensic analysis and genome sequencing. The process involves the amplification of distinctive chromosomal regions. Two oligonucleotides complementary to each of the DNA strands are used as primers for in vitro DNA synthesis. The sample is initially heated to separate the strands of DNA, upon cooling the primers anneal and therefore are ready for DNA synthesis. By repeating the cycle of heating and cooling the number of DNA strands grows exponentially and, for example, within 30 cycles it is possible to produce millions

of strands of identical DNA (Appenzeller, 1993). The original procedure involved the use of the Klenow fragment from Escherichia coli as the source of DNA polymerase. However, because the Klenow fragment is not thermostable, fresh enzyme had to be added during each cycle. From this initial procedure, PCR has developed and now a more specific and thermostable DNA polymerase, Taq polymerase, isolated from the thermophilic bacterium Thermus aquaticus is used. The technique is automated and has been modified in a number of different ways to enhance its efficiency (Erlich, Gelfand & Saiki, 1988), with the amplified sequence identified using electrophoresis or a number of other techniques. The whole process takes a few hours and is extremely sensitive.

PCR studies of sperm sex ratio are possible using Y chromosome- or X chromosome-specific primers. Studies using such PCR techniques have been criticized for being "many laboratory steps removed from an intact sperm" (Ericsson, 1994). However, it has been suggested that this criticism is due to the fact that studies using PCR have failed to confirm the success claimed by Ericsson for his albumin column selection technique (Martin, 1994). Ericsson's method for sex selection is probably one of the best-known techniques for selecting Y- bearing sperm. Since it was first suggested (Ericsson, Langevin & Nishino 1973), numerous studies have been carried out by various research groups with varying degrees of success. In the original study the ratio of X- to Y- bearing sperm was determined using quinacrine staining, however more recently a number of studies have used techniques such as FISH and PCR, which has further confused the issue of whether or not the technique is as successful as Ericsson claims.

Quinacrine fluorescent body (F-body) staining, devised by Barlow and Vosa in 1970, was the first method that appeared to enable the identification of Y- bearing sperm. The quinacrine stain is thought to intercalate into the DNA helix in heterochromatic regions that are abundant in adenine and thymine, such as the Yqh region on the Y chromosome. This results in an F-body that is much brighter than the background fluorescence and studies to prove the accuracy of the staining used interphase nuclei with XX, XY and XYY chromosomes that displayed none, one and two F-bodies respectively. The investigation of sperm populations using quinacrine staining produced varying results, however on average approximately 40% of sperm displayed one F-body, and on this basis they were considered to be Y- bearing (Barlow & Vosa, 1970; Bibbins, Lipshultz, Ward & Legator, 1988; Goldman Fowlina, Knights, Hill, Walter & Hulten 1993).

Considerable doubt has since been cast upon the reliability of quinacrine staining, by comparison with alternative, more sophisticated methods. One such study by van Kooij and van Oost (1992) comparing quinacrine staining with DNA probes, showed that sperm which were F-body negative did not correspond to X- bearing sperm when studied with DNA analysis. It has also been suggested that quinacrine staining stains heterochromatic regions on autosomal chromosomes. Because of these somewhat controversial results, quinacrine staining is no longer considered to be the most appropriate technique for determining the sex ratio of sperm (van Kooij & van Oost, 1992).

A number of investigators involved in sex preselection research, including Ericsson (1994), have stated that the best validation of any sex selection procedure is the resulting secondary sex ratio. There is however the need for some concern relating to this opinion, especially when discussing techniques

where the only successful results have been from *in vivo* studies. Laboratory analysis is important initially, it prevents the clinical use of techniques lacking a sound scientific basis and could be used to rule out any other possible variables affecting the result, such as the timing of insemination (Pyrzak, 1994). With *in vivo* studies it is impossible to rule out some form of sex selection occurring *in vivo*, and to determine the ratio of X- and Y- bearing sperm or the sex ratio at the time of conception. With the suggested spontaneous abortion rate being approximately 33%, the sex ratio at the time of conception could be very different (Zinaman, Clegg, Brown, O'Connor & Selevan, 1996).

2.7 Ethics of sex preselection

As previously stated sex preselection is a highly controversial subject, with certain groups in society calling for prohibitive legislation. Sex selection has however been part of many cultures for a very long time, even if it is primarily has taken the form of infanticide. Although infanticide as a form of sex selection cannot be regarded as ethically acceptable the use of other forms of sex preselection needs to be considered. Sex preselection must be divided into two forms, namely (i) when carried out for medical reasons, and (ii) for purely social reasons.

2.7.1.Sex-linked genetic disorders

There are sex-linked genetic diseases that cannot be prenatally detected, and in such cases, couples can elect either to select a child of the unaffected sex, or risk what often may be another affected child. Under these

circumstances, the method of sex preselection usually involves sex determination by either amniocentesis or chorionic villus sampling followed, if appropriate, by selective therapeutic abortion. Most medical practitioners agree that this method of sex preselection can only be justified under these circumstances and should not be used on solely social grounds. This attitude notwithstanding, sex preselection by amniocentesis even with the potential psychological and physical risks has been widely used in countries such as India and China (Shushan & Schenker, 1993).

PGD during *In Vitro* Fertilization (IVF) is slowly being adopted instead of sex determination followed by selective abortion. At present this technique is not legally permissible in Western Australia, and in many countries is only legal where there is a history of genetic disorders. There is however the potential for PGD to be used for social reasons. As discussed in chapter 2.2 there has been a recent case of a British doctor being publicly condemned for offering preimplantation sexing of embryos on social grounds in clinics outside the United Kingdom, to avoid the strict regulatory control of PGD that exists within the United Kingdom. The use of IVF and PGD is an expensive and complex invasive procedure, therefore even without regulation it has been suggested that its use on social grounds should be limited (Shushan & Schenker, 1993; Fletch, 1997).

The use of IVF in PGD and any other form of Artificial Reproductive Technology (ART) also opens up other avenues of ethical debate. Specific religious bodies have condemned such procedures, for example Pope Pius XII at the 2nd World Congress on Fertility and Sterility. The rationale behind the Papal condemnation of ART took the form of natural law, "on the subject of

attempts at artificial human fecundation *'in-vitro'*, let it suffice for us to state that such attempts must be rejected as immoral and absolutely unlawful" (Walters & Singer, 1982).

2.7.2. Preconceptional sex selection

Fortunately, most couples desiring a child of a particular sex tend to prefer the use of preconceptional sex selection. It has been shown that of couples inquiring about such techniques, a majority does not proceed with treatment. The reasons for this lack of uptake could be that the procedure is not 100% effective, that it may involve more than one insemination cycle, or the associated financial costs. It is perhaps the fact that the preconceptional methods are not of sufficiently proven efficiency that very few couples with medical reasons for sex selection decide to opt for their use (Jaffe, Jewelewicz, Wahl & Khatamee, 1991).

The majority of preconceptional methods involve the enrichment of X- and Y- bearing sperm. As with most forms of ART, a major ethical concern is the method of procuring the man's sperm. The method most commonly used is masturbation, which many societies have traditionally considered wrong. In recent years the stigma connected with masturbation has declined, but many groups still maintain the traditional view. For example, Jewish law forbids ejaculation outside the context of marital intercourse, because ejaculation such as by masturbation is regarded as akin to destruction of the seed. Under these circumstances, alternative methods of obtaining a sperm sample can be employed, for example, by coitus interruptus or the use of special non-toxic

condoms to collect the semen (Overburn & Fleming, 1980; Walters & Singer, 1982).

2.7.3. Effects upon society

It has been suggested that the use of sex preselection will lead to an increase in lawlessness, a lowering of moral standards, an increase in prostitution and male homosexuality, and a decline in marital norms. The basis for these judgements is that the use of sex preselection will alter the naturally occurring secondary sex ratio. However, changes to society of this nature have not been observed in areas where there is already an unbalanced tertiary sex ratio, such as Alaska which has 132 males per 100 females (Rueggsegger Veit & Jewelewicz, 1988). The theory that the sex ratio will in fact be altered is also a source of debate. A basic requirement of sex selection is the need for pregnancy planning. This is however something that many couples at present appear to find well-nigh impossible, with reports of up to 50% of pregnancies being unplanned.

Nevertheless, there does appear to be a need for the application of some form of regulation to sex preselection. The majority of articles published on this topic state that the final decision should be left to individual couples. There might however need to be some guidance provided, for example, along the lines suggested by Pennings (1996):

a) Sex selection is not allowed for the first child.

b) Sex selection is not allowed when there is a balance of sexes in the family.

c) The sex selected can only be the lesser-represented gender in the family.

d) The above guidelines are overruled if the prevention of a child of a certain sex is advisable on the basis of increased genetic risk.

Some regulation placed on clinics offering sex preselection might also be beneficial to ensure that those seeking the procedure would receive a high quality of service, and preventing unscrupulous investigators offering unproven techniques, especially since regulation of this nature is already in place for most other ARTs (Editorial, 1993; Shushan & Schenker, 1993; Pennings, 1996).

2.8 Aims of this study

The primary aim of the study was to evaluate two different methods of enriching X- and Y- bearing sperm, using fluorescent *in situ* hybridisation to determine the final ratio of the two sperm types. The two methods to be used were Human serum albumin columns and ISolate® discontinuous density gradients through which the sperm samples were passed. The samples used were from men who have fathered three or more children who are all of the same sex. It was envisaged that the methods will permit determination of whether these subjects had an altered ratio of X- to Y- bearing sperm in their semen, and the value of the sperm enrichment methods to preferentially enrich either X- or Y- bearing sperm.

The answers to the following research questions were being sought by this study:

1. An evaluation of dual colour fluorescence *in situ* hybridisation as a method of sexing sperm.
2. To determine if men with children who are all of the same sex, have semen with an altered X-to Y- bearing sperm ratio.
3. To investigate the use of Human serum albumin columns as a method of enhancing the proportion of either X- or Y- bearing sperm in the final sample.
4. To study the use of ISolate® discontinuous density gradients as a method of altering the proportion of either X- or Y- bearing sperm in the final sample.

3.1 Semen Analysis

3.1.1 Standard Semen Analysis

Semen is comprised of sperm suspended in secretions from the testis, epididymis, prostate, bulbourethral glands and seminal vesicles. Van Leeuwenhoek first noted the presence of sperm within semen in 1679, using the newly discovered technique of light microscopy (Mittwoch, 1985). Seminal fluid contains a number of compounds including fructose, which acts as an energy source for the sperm, prostaglandins, which stimulate muscle contraction within the female reproductive tract thus aiding the movement of the sperm towards an oocyte, vitamin C, zinc and cholesterol. The secretions from the seminal vesicle and prostate gland are alkaline and so help to neutralize the other secretions which are slightly acidic. The alkalinity of the semen also helps to neutralize the acidic secretions of the female reproductive tract.

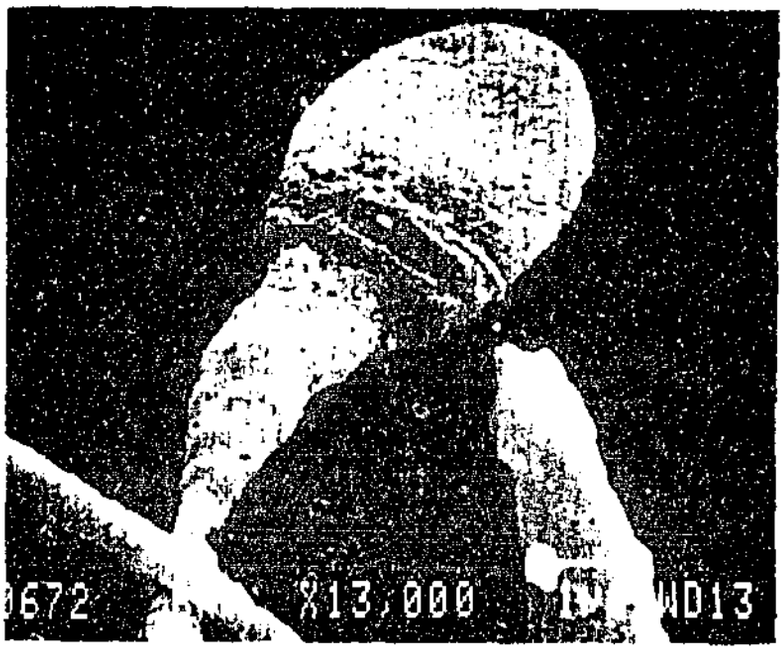
Sperm are not evenly distributed throughout the ejaculate. Approximately 80% of sperm are found in the first 20% of the ejaculate, the remaining 80% being predominantly secretions from the seminal vesicles. The secretions produced by the various glands are mixed immediately prior to ejaculation (Tyler, n.d).

Populations of sperm normally have variable morphology. From the examination of sperm recovered from the female reproductive tract following coitus, a number of different definitions of normal sperm has been suggested. For example the Strict classification system which was originally described by Kruger et al (1986). This classification system defines a normal sperm as having a smooth, oval shaped head with a well-defined acrosome. The sperm

head must also be between 5.0-6.0μm long and 2.5-3.5μm wide, with no head or other defects. This classification system requires that any boarderline forms be considered abnormal, due to this, normal men score approximately 14% normal forms using this scoring system.

Due to the different morphological classification systems, the World Health Organization produced a system, which incorporated the best aspects of several different systems. A normal sperm, such as that shown in Figure 3.1, has been described as having an oval shaped head, which is 3.0-5.0μm long and 2.0-3.0μm wide with a tail, which is cylindrical and between 45-50μm long. Using this system, in a normal semen sample at least 30% of the sperm will confirm to the criteria.

Figure 3.1: Scanning Electron Micrograph of a normal sperm (Hollanders et al, 1996).



Any sperm that does not fit the description of a normal sperm is classified as abnormal and categorized according to the nature of the abnormality. The general groups of defects include:

Table 3.1: Sperm abnormalities

Defect	Examples
Head shape and size	Large, small, tapering, pyriform, amorphous, vacuolated and multiple heads.
Neck and midpiece defects	Non-inserted or bent tail, distended/irregular/bent midpiece, midpiece with no mitochonfrial sheath (thin) and absent tail (free or loose head).
Tail defects	Short, multiple, hairpin, broken, irregular width, coiled tails and tails with terminal droplets, (see Figure 3.3).
Cytoplasmic droplets	Classified when one-third the size of a normal sperm head.
Headless (pinhead) sperm	(see Figure 3.2)

(World Health Organisation, 1992).

Figure 3.2: Sperm morphological defect - pin head

(Hollanders *et al,* 1996)

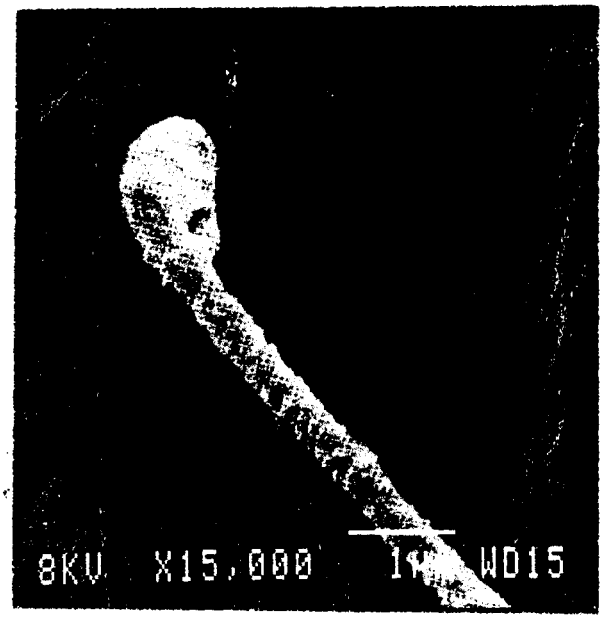
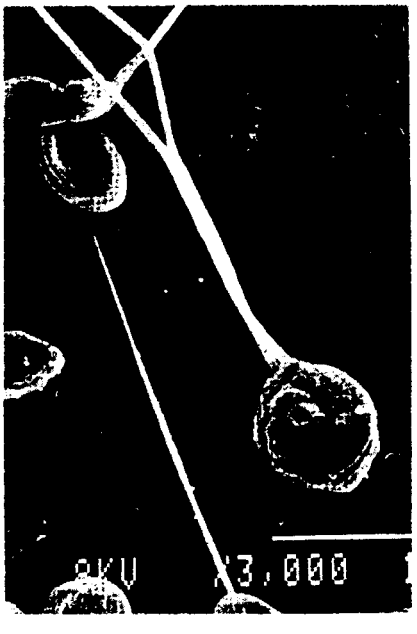


Figure 3.3: Sperm morphological defect - tail

(Hollanders *et al,* 1996)



Semen analysis was first described as a method of evaluating the possibility of a male factor in infertile marriages in the 1920s (Macomber, 1929). This initial analysis involved a count of the total number of sperm present, and therefore gave very little indication as to the fertility of the males involved. Studies such as that by MacLeod in the early 1950s provided more information on male infertility, by comparing the sperm counts of men with proven fertility with those from infertile marriages (MacLeod, 1951; MacLeod & Gold, 1951). From this study, it was deduced that a count of 20 million/ml was the minimal number of sperm required for normal fertility. Like many subsequent studies, the work of MacLeod has been criticized for several reasons, for example, the use of a possibly unsuitable control group and failure to control the period of sexual abstinence before the analysis was conducted (Lipshultz, 1982). More recent studies based on fertile men presenting for vasectomies have suggested that 10 million/ml might be a more appropriate figure (Smith & Steinberger, 1977).

In 1980 the World Health Organisation (WHO) published a Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction. The manual set out standard procedures for semen analysis, with the intention of improving the precision and reproducibility of semen analyses carried out in andrology laboratories worldwide. The WHO manual is now in its third edition and the revisions that have been incorporated highlight the rapid advances made within the field of andrology. A standard semen analysis in accordance with the WHO recommendations consists of measurements of semen volume, appearance, consistency, and pH, and sperm density, motility,

progression and morphology. Each of these c aracteristics are discussed in more detail within the methodology.

There are a number of other tests that can be carried out to provide further information on the quality of the semen sample. The aim of these tests are to more fully investigate specific sperm abnormalities, diseases of the male reproductive system and function of the sperm. A summary of such tests is given below:

Table 3.2: Additional semen analysis tests

Test	Explanation
Vital staining	The sperm vitality is the proportion of sperm that are alive as determined by staining. This test can be useful when there are more than 50% immotile sperm in a sample.
Antisperm antibodies (ASABs)	This test can indicate if there is a possible immunological explanation for infertility.
Peroxidase staining	Allows differentiation between white blood cells and immature sperm calls. Can be carried out when there appears to be a high leucocyte count which may indicate the possibility of infection.
Semen culture	Test for bacteria within a semen sample; their presence can suggest a genital infection.
Hypo-osmotic swelling test	This assesses the sperm membrane for structural integrity; the test is an alternative method by which sperm vitality can be assessed.
Biochemical analysis of semen	Measures the levels of chemicals, such as fructose, that are essential for sperm function. For example, the absence of fructose would indicate the absence of seminal vesicles or an obstruction of the ejaculatory ducts.
Hormone evaluation	Measurement of the blood levels of hormones that are known to influence sperm production, for example, follicle stimulating hormone and testosterone.
Post-coital/ cervical mucus test	To assess the compatibility of a man's sperm with the mucus of his partner's cervix.
Sperm penetration assay	Measures the ability of a man's sperm to penetrate an egg. This test is usually only undertaken prior to IVF and is not available in Australia because it requires hamster eggs (see section 2.2)
Human zona pellucida binding test	Determines the ability of sperm to bind to the zona pellucida of the egg. Can be used instead of the sperm penetration assay when the use of hamster eggs are not permitted.

Sources: (American Society of Reproductive Medicine, 1997; World Health Organisation, 1992; Mortimer, 1994b)

3.1.2 Clinical value of semen analysis

As previously stated, the purpose of the World Health Organisation manual on semen analysis was to standardize the analysis of semen samples. However, there have been concerns raised about the accuracy of the tests carried out and therefore their clinical value. A number of studies have been conducted in order to assess the external and internal quality control of andrology laboratories (Neuwinger, Behre & Nieschlag, 1990; Cooper, Neuwinger, Bahrs & Nieschlag, 1992). These studies have shown considerable inter-technician and inter-laboratory variation in the results of semen analyses. The degree of variation observed by Neuwinger and colleagues (1990) was 38% which is considered unacceptable, with a value less than 20% regarded as more acceptable (British Andrology Society, as cited in Jequier & Ukombe, 1983). The two main reasons given for the high degree of variation are firstly, the fact that the majority of andrology laboratories do or did not follow a standard training procedures, and secondly the variation in methods used to perform the semen analyses. Thus it is quite common for technicians from the same and different laboratories to use different methods to conduct what should be a standard semen analysis. Both of these factors were in theory addressed by the publication of the World Health Organisation manual, and the establishment of a number of training courses, for example, those run by the British Andrology Society (Barratt, 1995).

Due to the considerable variation in the results of semen analyses carried out by different andrology laboratories, it has been suggested that conventional semen analyses provide very little predictive information, and therefore they may not be as important as diagnostic indicators as previously thought (Dunphy,

Neal & Cooke, 1989). For example there is considerable controversy over the clinical significance of abnormal sperm morphology, although it has been accepted that a high incidence of abnormal forms is associated with reduced fertility. The World Health Organisation has stated that 30% of sperm within a sample should be of normal forms, but this value has not been evaluated in clinical studies.

However, a study by Hargreave and Elton (1983) stated that although semen analyses provided limited information, they should be carried out. It was found that the sperm count and motility of a semen sample were reasonably satisfactory indicators of fertility, and so until more objective methods of assessing semen were available the standard semen analysis as described above will probably continue to be integral component of any investigation into the likelihood a couple will conceive.

Even simply using sperm counts and motility's there is still debate about these results and their significance, for example, a study carried out by Dunphy and colleagues (1989) studied the significance of the different grades of sperm in terms of potential fertilizing capacity. It had been assumed that the grade a sperm were the most important for fertility, however Dunphy, Neal and Cooke (1989) stated that perhaps it was the grade b sperm which were the most important. One possible explanation for this finding is that very rapid sperm velocity could result in depletion of the intracellular ATP and hence decrease spermatozoal longevity (Dunphy, Neal & Cooke, 1989).

3.1.3 Computer-assisted semen analysis

It is now possible to evaluate a number of the parameters of a semen sample using automated methods. Sperm concentration can be measured using a counter such as the Coulter Counter. However, the presence of other cells and debris can reduce the accuracy of the count, as can the presence of low sperm concentrations. Sperm motility can also be assessed using a direct digitization of the electrical signal originating from a video scan of a microscope field containing sperm. By continuously monitoring the movement of individual sperm it is possible for the computer to calculate the number of sperm displaying specific motility patterns, such as the percentage of sperm swimming progressively and in a linear fashion (Parikh, Kamat, Kodwaney & Balaiah, 1996). Stained smears of semen can also be evaluated using videomicrography to assess sperm morphology. The sperm can be recorded on to video at high magnification, with the image of the sperm projected on to a video screen with a transparent overlay of standard sperm head length and width (Overstreet & Katz, 1987).

The use of computer-assisted semen evaluation is limited by the lack of established "normal" values of any for the quantitative parameters tested. Until there are sufficient clinical data on each of the semen parameters, computer evaluation will remain limited by the fact the result will still be subject to the personal interpretation of the operator. Over the past ten years there have been vast improvements in computer assisted semen analysis (CASA) systems available. There are now a number of different systems available, some of which have been integrated into routine use within a number of andrology laboratories. It has been suggested that there might be significant differences

between the results obtained from a computer system and an experienced andrologist (Hofman, Santilli, Kindig, Scott & Johnson, 1996). However, considering the fact that a number of studies have observed both intra- and inter-technician differences, it could be expected that there would be differences between the results from a CASA system and those of an andrologist.

3.2 Human serum albumin gradients

The use of bovine serum albumin (BSA) or ovalbumin-containing medium to isolate fractions rich in human Y-bearing sperm was first suggested over twenty years ago (Ericsson, Langevin and Nishino, 1973). The basis for this form of isolation was the differential motility exhibited by X- and Y- bearing sperm. It was claimed that the enrichment process produced fractions containing up to 85% Y-bearing sperm, of which 93% to 98% were progressively motile.

Ericsson and his colleagues suggested different possible methods using BSA-containing media. All of the columns were produced in Pasteur capillary pipettes that were heat-sealed at the point of taper, and were overlaid with a standardized preparation of 50 x 10^6 sperm in 0.5 ml of Tyrode's solution. The first method consisted of basic columns with BSA concentrations of between 3% and 25%. The sperm samples were overlaid on to the columns and incubated for 1 hour at room temperature before being removed by aspiration. The second approach involved the use of replicate columns consisting of 6%, 10% and 20% BSA. This technique required the recovery of motile sperm by centrifugation, followed by transfer on to the next column. The approach was

simplified in the third procedure which utilized a single column containing multiple layers of different concentrations of albumin. The fractions of isolated motile sperm were washed and prepared for quinacrine staining, which was used to determine the concentration of Y-bearing sperm in the neat semen and in each of the processed fractions, with 600 sperm counted in each of the samples.

The most successful Y-enrichment was obtained using a single column containing three layers of 10%, 15% and 25% BSA. With this technique, it was claimed that 85% of sperm were Y-bearing, 98% of which were motile (Ericsson, Langevin & Nishino, 1973). The other methods also enhanced the proportion of Y-bearing sperm in the final fraction, although in these cases the levels achieved were between 57% and 76% (Ericsson, Langevin & Nishino, 1973). Following the publication of these results, numerous research groups undertook studies based on the BSA-based enrichment methods. The studies undertaken can be divided into two groups, those which used laboratory analysis to determine the percentage of Y-bearing sperm and the remainder which relied on determination of sex ratio of the offspring following insemination.

From the data of successful studies, it has been suggested that an important factor is the percentage of motile sperm recovered from the initial total motile sperm (TMS). Studies such as that by Beernink et al. (1993) had recoveries of motile sperm that were less than 10% of the initial TMS. By comparison Wang et al. (1994a) did not observe any enrichment of Y-bearing sperm, but they did report motile sperm recoveries in excess of 10%, in this case 16-19%. Pyrzak (1994) suggested that a "recovery of >10% of TMS appeared to increase the number of X-bearing sperm..... the lower the recovery,

the better the chance of getting male offspring." The samples containing less than 10% of the original TMS usually displayed very high sperm motility and strong velocity. They also did not contain extraneous particulate material, including germinal and epithelial cells and other debris (Gledhill, 1988b).

Most of the BSA-based studies employ methodologies that are described as being modified from the Ericsson protocol and it seems probable that such methodological variation means the results cannot be considered as directly comparable with the original study. For example, a change in the temperature of incubation from room temperature to 37°C would be expected to significantly increase the sperm velocity (Vidal et al., 1993; Ericsson, 1994). Other modifications include a variation in the dimensions of the tubes in which the columns, are formed or different media to produce the HSA solutions. The original methodology used by Ericsson, Langevin and Nishino (1973) was based on columns using BSA. A common modification is the use of human serum albumin (HSA) for column preparation, because albumin from animal sources cannot be used if the final fraction is to be used for human insemination. The use of HSA has been included in the HSA procedure which is licensed by Gametrics.

As previously noted, many of the studies using laboratory-based analysis to determine the percentage of Y-bearing sperm employed quinacrine staining. As discussed in chapter 2.6 this method has since been shown to be unreliable (van Kooij & van Oost, 1992), and other methods have become more popular, for example, fluorescence *in situ* hybridisation (FISH) and polymerase chain reaction (PCR). Many insemination studies did not employ untreated, control groups, the usual reason being that the secondary sex ratio, i.e., the sex ratio at

birth, is well established. However the possibility that the claimed success of the studies is a result of a number of compounding factors needs to be considered (Martin, 1994, Beernink, Dmowski & Ericsson, 1993). Therefore in all insemination studies, ideally there would be two randomized groups, both containing couples desiring a child of a particular sex. One group of women would be inseminated with the HSA-gradient processed sperm of their partner while in the other, the women would be inseminated with washed sperm (Carson, 1988).

This method of sperm separation has been patented by Ericsson and franchised by Gametrics Limited (Carson, 1988). Since 1975 some 1500 children have been born in over 65 centres which use an albumin enrichment method (Ericsson, 1994). An example of such a centre is the clinic opened in Utrecht, Netherlands in the summer of 1995. The clinic offers the HSA gradient sperm separation technique to married couples wanting to select the sex of their second or subsequent child. There were a number of criticisms of the clinic when it opened, especially on the grounds of the enrichment technique used. As cited in Sheldon (1995), a spokesperson for the Dutch In Vitro Fertilization Association noted: "This technique is very controversial. It does not mean it does not work but there is no objective scientific data. If Dr. Ericsson really wants his technique accepted as scientific truth there needs to be a prospective controlled study by an independent authority." However Ericsson has not deemed this necessary (Sheldon, 1995).

3.3 ISolate® discontinuous gradients

Studies carried out by Kaneko and his colleagues in Japan in the 1980s showed that X-bearing sperm sediment faster than Y- bearing sperm in discontinuous density gradients (Kaneko, Yamaguchi, Kobayashi & Iizuka, 1983). Using 12-step Percoll gradients, samples containing up to 94% X-bearing sperm were isolated. The exact mechanism for this form of enrichment is not currently understood. A theory that has been suggested is that the sperm separate due to differences in their differential velocity of sedimentation. Another suggestion is that X- bearing sperm have a greater nett negative charge on their surface, and so an interaction between the sperm surface and the medium might influence separation (Gledhill, 1988b; Wang, Flaherty, Swann & Matthews,1994a).

A common methodology used in Percoll-based studies is that of Iizuka et al. (1987). Combining 9 parts of Percoll with 1 part 1.5M NaCl buffered with 0.1M HEPES makes the standard Percoll solution. The solutions for use in the columns contain Percoll concentrations between 30% and 80% in 5% intervals, and these solutions are produced by diluting the standard Percoll solution with Hams F10 medium buffered with 10mM HEPES. The gradients are formed in round bottom culture tubes and consist of 0.7ml of each Percoll solution, with the sperm suspension layered on top. The gradient is centrifuged at 250g for 30 minutes, after which the upper layers are aspirated or the 80% layer, which contains the X-bearing sperm, is removed through a hole in the bottom of the tube. The 80% Percoll layer is centrifuged and the resulting sperm pellet diluted to the required concentration (Wang, Flaherty, Swann &Matthews 1994b).

The original Percoll studies determined the ratio of X- to Y- bearing sperm using quinacrine staining. Alternative methods now considered to be more reliable have also been used to evaluate the technique, for example Southern blotting and FISH but these methods have failed to confirm enrichment of up to 94%. For example, Wang et al. (1994b) using FISH did observe X-bearing sperm enrichment of up to 57.2%, whilst van Kooij and van Oost (1992) did not detect any enrichment in their DNA-probe studies. Some investigators have also commented on the fact that this technique usually results in low recovery rates and slight decreases in sperm motility (Zarutskie, Muller, Magone & Soules, 1989). Van Kooji and van Oost (1992) demonstrated that Percoll interfers with quinacrine staining, thus possibly explaining the low percentage of Y- bearing sperm in Iizuka *et al* (1987) results.

The use of Percoll is however only licensed for research purposes and not for sperm isolation for the purpose of Assisted Reproduction technologies. This limitation is imposed because the manufacturing process utilizes quality control procedures at levels required for other than in vitro use. For that reason, a number of companies have developed products specifically designed to replace Percoll, for example Nycodenz and ISolate® (SIRT Quarterly, Summer 1996).

ISolate® is a "sterile colloidal suspension of silica particles stabilized with covalently bound hydrophilic saline in a HEPES-buffered system" (Irvine Scientific, undated). Product testing by the manufacturer Irvine Scientific shows that ISolate® gradients produce motile concentrations, percentage yields and 24 hour motility and progression scores that are comparable to Percoll gradients.

Standard cytotoxicity testing has shown that ISolate® is non-cytotoxic and is biologically inert (Irvine Scientific, undated).

3.4 Fluorescence *in situ* hybridisation

Fluorescence *in situ* hybridisation (FISH) was developed from isotope *in situ* hybridisation and it was first employed to hybridise the Y chromosome in ejaculated sperm using specific probes (Guttenbach & Schmid, 1990). FISH is one of several forms of non-isotopic ISH that were developed because of the potential radiation problems associated with the use of isotopes. FISH is becoming increasingly popular in determining the sex ratio of sperm. It is a cytogenetic technique that uses non-radioactive commercially available fluorescence-labeled DNA probes, which are specific to certain chromosome regions. The technique is highly sensitive and so it can be used on both meta- and interphase cells (Pettenati, Nagesh-Rao, Schnell, Hayworth-Hodge, Lantz & Geisinger, 1995). Dual colour FISH was developed to improve the reliability of the technique by reducing the risk of erroneous results. Previously, when only a single probe against either the X or Y chromosome was used, it was impossible to determine if unlabelled sperm contained the other chromosome or if the probe was simply unable to enter the nucleus. By using specific probes against X and Y chromosomes, both X- and Y-bearing sperm can be detected, as can the number of unlabelled sperm (Han, Ford, Webb, Flaherty, Correll & Matthews, 1993).

One of the problems associated with this form of technique is the need to decondense the sperm nuclei before hybridisation can occur. Sperm nuclei

become highly condensed during spermatogenesis, when the nuclear proteins are replaced with protamines. Protamines are sperm-specific, arginine-rich nuclear proteins that neutralise the DNA phosphate charges and form a network consisting of both intra- and inter-protamine disulphide bridges. A number of different reducing agents have been used, the most popular of which involves the use of dithiothreitol (DTT) (Wyrobek, Alhborn, Balhorn, Stanker & Pinkel, 1990). DTT decondenses the sperm nuclei, but the sperm membranes are left intact thereby allowing ready identification of specific sperm types.

As previously noted many of the probes used are commercially available and they are supplied with specific protocols for use. In general, following decondensation and fixation with methanol:acetic acid (3:1), the sperm are dehydrated using ethanol and the probes applied. Hybridisation usually requires between 18 to 24 hours, during which time the slides are normally kept in a humidified environment to prevent drying. The fluorescent probes are then applied and there are often amplification steps involved to ensure a good signal. Once this has been completed, the slides can be examined under fluorescence microscopy.

Laboratory analysis should be an important prerequisite, this would serve to prevent the clinical use of techniques with no sound scientific basis, and rules out other possible variables affecting the result, such as the timing of insemination (Pyrzak, 1994).

4. Subjects and experimental methods

4.1 Study design and development

The study was divided into three sections, the initial investigations, pilot study and clinical subjects. The inital investigations were used to establish enrichment protocols which fulfill the predetermined requirements, ie., that the final sperm fractions contained less than 10% of the inital total motile sperm and that at least 90% of the sperm were motile. The FISH inital studies were used to identify a protocol for dencondensing the sperm head, which produced samples that consisted of at least 90% decondensed sperm heads.

The pilot study was used to test that the protocols established in the initial investigations. The sperm samples were processed by either the HSA columns or ISolate® and then processed by FISH. The purpose of the pilot study was to indentify any problems with the combining of the sperm processing with the FISH techniques.

The donors for this study were recruited as described in chapter 4.2, the samples from these donors were processed using the protocols established and confirmed in the initial investigations and the pilot study.

4.2 Recruitment of donors

The donors were recruited into the study following the publication of an article in The West Australian (Amalfi, 1997), which attracted considerable publicity in the Perth Metropolitan region. A journalist from The West Australian wrote the article after a meeting with Dr. Matson and myself arranged by the

Public Relations Department of King Edward Memorial Hospital. In brief, the article (see Appendix A) requested that men with three or more children of the same sex who were interested in the study should telephone Dr Matson at Concept Fertility Centre.

Prospective donors who made contact were invited to Concept Fertility Centre to discuss the study in greater detail, and they also were given a copy of the Information sheet (see Appendix B). The donors were informed when they expressed an interest in the study that there would be the need for them to produce a semen sample by masturbation at Concept Fertility Centre, where special facilities are available. Those who decided to proceed were asked to sign a Consent form (see Appendix C), and arrangements were made for them to produce a semen sample at Concept Fertility Centre. All donors were asked to contact Dr. Matson approximately one week later to arrange an appointment for the collection of a second sample.

When donors came to Concept Fertility Centre to provide a sample they were asked to complete the top section of a Concept Semen Analysis sheet (see Appendix D) and given the standard Concept instructions concerning semen collection. Immediately on receipt in the Andrology laboratory, the semen sample was placed in a 37 °C incubator for 20 minutes to allow the sample to liquefy.

Semen samples from donors to Concept Fertility Centre were used for the initial studies to establish the techniques involved in the formation of the protocols for the ISolate® gradients and Human serum albumin columns. The semen samples from the study donors who had three or more sons were

processed using discontinuous ISolate® gradients, while semen samples from the donors with three or more daughters were processed using Human serum albumin columns. The semen samples were processed in this manner because it is these groups which are most likely to request these types of enrichment, i.e. those with three or sons requesting X- bearing sperm enrichment and vice versa. From each semen sample a control aliquot was washed (as described in chapter 4.2.9) and prepared for fluorescence *in situ* hybridisation, thus making it possible not only to determine the degree of enrichment observed but also whether the donors had an altered ratio of X and Y- bearing sperm. When a second sample was available from a donor, it was processed in an identical manner to the first, to determine if the enrichment procedure was reproducible.

4.3 Semen Analysis

The semen analysis was carried out within one hour of production of the sample, and usually within 30 minutes. The results of the analysis were recorded on the Concept Semen Analysis sheet (see Appendix D), which was kept at Concept. For any samples or documentation that were removed from Concept Fertility Centre, the donors and the samples were identified by unique codes.

4.3.1 Appearance

The appearance of the semen sample was recorded as being either normal or abnormal according to the following criteria. A normal semen sample has an even, grey-opalescent appearance and may contain gelatinous bodies

that do not liquefy. The sample may not appear particularly opaque, which can be associated with a low sperm concentration. Contamination of a semen sample with urine results in a slight yellow discolouration, a distinctive odour and very low or no sperm motility due to the toxic effects of the urine. Blood contamination has a number of different appearances depending on the volume and age of the blood involved. A pink colouration indicates the presence of fresh blood, whereas old blood, such as that from bleeding which occurred hours or days before, results in a brown appearance. It has also been known for bilirubin to contaminate the semen, resulting a bright yellow sample (Mortimer, 1994b; World Health Organisation, 1992).

4.3.2 Volume

The volume of the whole ejaculate is measured by aspiration into a 5ml or 10ml wide-mouthed pipette, depending on the size of the sample. Plastic syringes and hypodermic needles are not used because they have been shown to reduce the motility of the sperm. Between 2 ml and 5 ml is considered the norm, with any extreme variation from this thought to have an effect on the transport of sperm through the female reproductive tract (British Andrology Society, undated).

4.3.3 pH

The pH of the semen sample tends to increase with time, hence the pH was determined as soon as possible after the semen has liquefied in vitro usually within 30 minutes of ejaculation and certainly within an hour. The pH was determined using Whatman pH 6-8 paper and should be between 7.2 and

8.0; any variation from this range could be an indication of inflammatory disorders of the prostate or seminal vesicles (Mortimer, 1994b).

4.3.4 Consistency

The consistency is also often referred to as the viscosity of the liquefied sample. The consistency was determined by allowing the semen to drop from a wide-mouthed pipette. Normal semen drops in small discrete drops, while a sample with abnormal consistency forms threads, usually greater than 2 cm in length. An abnormal consistency can make determination of the sperm motility and count almost impossible. Highly viscous semen can reduce sperm motility and may be a significant factor contributing to a failure of fertilisation (World Health Organisation, 1992; British Andrology Society, undated).

If the consistency was abnormal the sample was mixed with an equal volume of Earle's culture medium (described in Appendix F), by repeatedly drawing the sample up into a 1ml Pasteur pipette until thoroughly mixed.

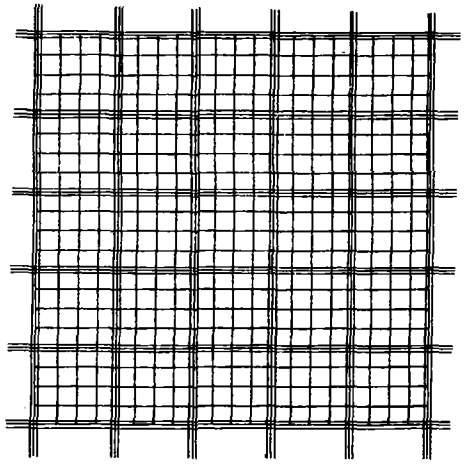
4.3.5 Total and Motile Sperm Count

There are a number of different counting chambers available for sperm counting. From the literature, one of the more popular chambers appears to be the Makler chamber from Sefi Medical Instruments, although it has been suggested that chambers such as this are not as accurate as hemocytometers and therefore should not be used (Mortimer, 1990).

0.1ml of the sample was diluted into 0.9ml of water, and a second 0.1ml of sample was diluted into 0.9ml of Earle's Flushing Media (see Appendix F). The dilutions were gently mixed to ensure even sperm distribution. A

hemocytometer coverslip was fixed over the chamber and a drop of the semen diluted in water was placed into the hemocytometer using a microhaematocrit tube. To check that the hemocytometer coverslip was correctly fixed and therefore that there was a uniform volume of liquid within the chamber, the coverslip was examined for the presence of "Rings of Newton". The hemocytometer was observed under a Olympus CH microscope at x100 magnification and the number of sperm within three separate rows of five squares (as shown in Figure 4.1) were counted. Only morphologically mature germinal sperm cells with tails are counted, with "pin-head" sperm and tailless heads excluded.

Figure 4.1: Central squares from Neubauer hemocytometer (Mortimer, 1994b)



The mean of these counts, was used to calculate the total number of sperm in millions per millilitre, according to the appropriate correction factor shown below:

Table 4.1: Correction factors required with the Neubauer hemocytometer

Sample	Haemocytometer Correction Factor
Semen dilution	Divide number of sperm counted in 5 squares by 2
Final sperm fractions following either ISolate® or Human serum albumin treatment	Divide number of sperm counted in 5 squares by 20

As previously stated, although a sperm count is one of the oldest forms of semen analysis there is still considerable disagreement on the level at which Oligozoospermia, which is the nomenclature used to described semen samples containing low sperm counts, should be set. At present the World Health Organisation states that Oligozoospermia exists when a sample contains less than 20 million/ml (Mortimer, 1994b; World Health Organisation, 1992), i.e., the figure originally proposed by MacLeod (1951).

The hemocytometer was then wiped clean and the coverslip was refixed over the chamber. A drop of the semen diluted in Earle's Flushing Medium was placed into the hemocytometer and the number of nonmotile sperm in three rows of five squares was counted to allow the calculation of the number of motile sperm.

4.3.6 Sperm Progression

Whilst still using the hemocytometer containing the semen diluted with Earle's Flushing Medium, two groups of 100 sperm are examined under light microscopy and graded according to the pattern of motility they display. The World Health Organisation (1992) classification system is:

Table 4.2: WHO sperm progression classification system

Grade	Explanation
a	Rapid progessive motility
b	Slow or sluggish progressive motility
c	Nonprogressive motility
d	Immobility

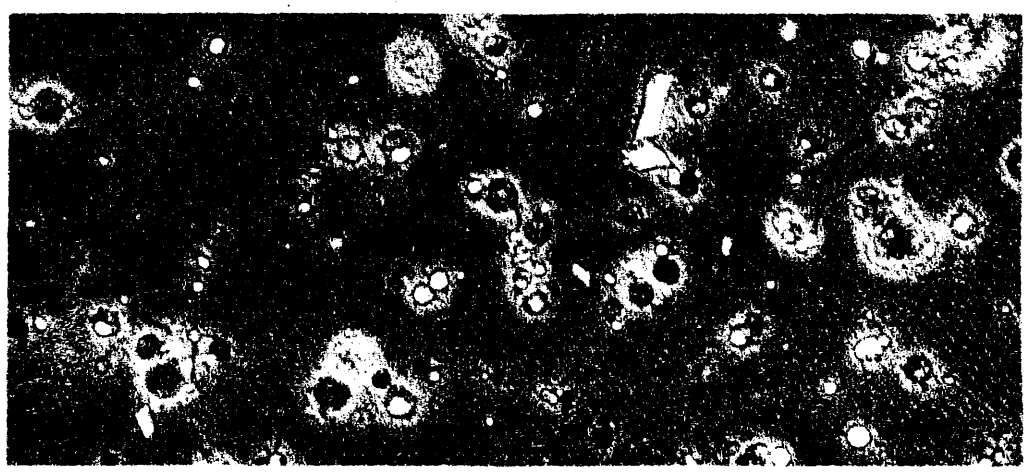
The average values obtained with the two groups are expressed as percentages, to describe the motility of sperm within the sample. Normal semen samples contain at least 50% motile sperm (grades a, b and c) and usually approximately 40% progressively motile sperm (grades a and b) (Mortimer, 1994b; World Health Organisation, 1992; British Andrology Society, undated).

5.3.7 Presence of cellular material other than sperm

It is considered quite normal for semen to contain cells other than sperm. The types of cells routinely present include polygonal epithelial cells from the urethral tract, spermatogenic cells and leucocytes. The presence of a large number of leucocytes, known as leucocytospermia can, however, indicate an infection within the reproductive tract and therefore noted. Leucocytospermia can result in a reduction in sperm concentration and motility due to oxidative stress and/or the secretion of cytotoxic cytokines. As a rule it has been suggested that a normal ejaculate should not contain more than 1 million/ml leucocytes, however it has proven difficult to determine the concentration of leucocytes that would begin impacting upon fertility. The presence of a very

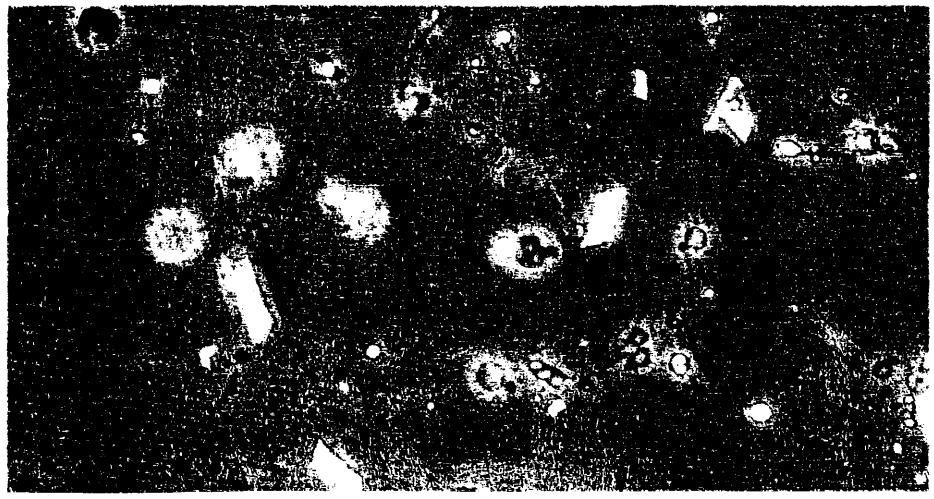
large number of leucocytes may also be accompanied by bacterial or protozoal contamination. Bacteria and protozoa are not normally present in semen, and hence their presence is usually associated with an infection at some site within the male genital tract (Mortimer, 1994b).

Figure 4.2: Semen samples containing bacteria and leucocytes (Mortimer,1994b)



Quite commonly particulate debris is observed within semen, defined as cellular material that is smaller in size than a sperm head. There is no objective manner in which the presence of debris can be scored, however, the presence of heavy debris contamination is considered significant.

Figure 4.3: Semen samples containing high levels of particulate debris (Mortimer,1994b)



Semen samples obtained via coitus interruptus invariably contain high numbers of vaginal epithelial cells. Samples collected using non-toxic condoms often contain higher levels of epithelial cells, possibly due to exfoliation from the urethra or glands (Mortimer, 1994b; World Health Organisation, 1992).

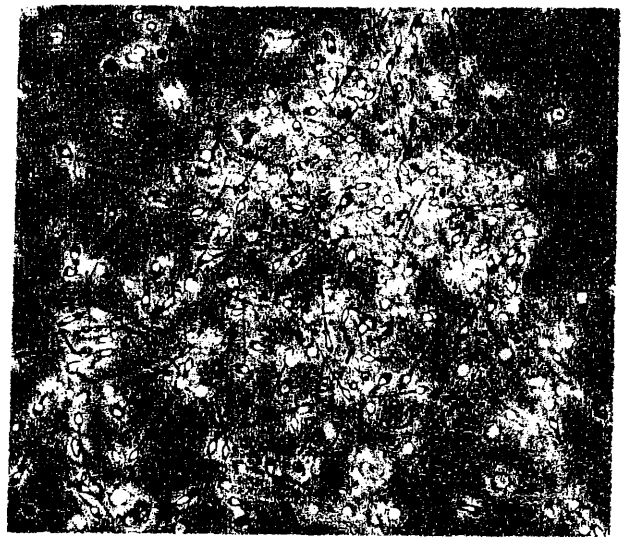
4.3.8 Agglutination

Agglutination is the specific binding of sperm via antibodies. Sperm usually adhere to each other via three sites, at the head, midpiece or tail. The manner of binding, whichever that be, head-to-head, midpiece-to-midpiece, tail-to-tail, tail tip-to-tail tip or mixed, such as head-to-tail, is thought possibly to be significant in terms of fertility. The percentage of sperm bound into clumps is used as an indicator of the possibility of an immunological cause for infertility and hence agglutination of sperm within a sample was noted. The presence of

antisperm antibodies (ASABs) can be assessed using a mixed antigolgulin reaction. ASABs are thought to occur in approximately 10% of infertile individuals (Matson, 1994) and they appear to exert a very complex influence on infertility. ASABs have been shown to influence sperm motility, cervical mucus penetration, sperm-zona pellucida interaction and the acrosome reaction.

Agglutination only involves sperm, and any clumps containing other cells or debris are identified as non-specific aggregation. In agglutination the majority of sperm are usually motile whilst this is not the case with non-specific aggregation. In both cases however, the motility of the sperm is reduced which in itself is a significant factor in sperm fertilising ability (Mortimer, 1994b; World Health Organisation, 1992).

Figure 4.4: Examples of sperm agglutination (Mortimer,1994b)



4.3.9 Preparation of a washed sample

For both the initial studies of ISolate® discontinuous gradients and Human serum albumin columns, neat and washed semen was used. The washed semen was prepared using standard Concept andrology laboratory washing procedures. For washing a sample of semen, 1ml of semen was diluted with 2ml of Earle's Flushing Medium and gently mixed to ensure an even suspension. If the semen had an abnormal consistency it was sometimes necessary to mix the semen with the medium by repeatedly drawing the sample up into a 1ml Pasteur pipette. The suspension was then spun in the Hettich Zentrifugen Universal 16A centrifuge in the Andrology laboratory at 2100rpm for 7 minutes, the supernatant was removed and the pellet was resuspended in 1ml of Earle's Flushing Medium. The sperm pellets removed from an ISolate® discontinuous gradient or Human serum albumin column were resuspended in 1ml of Earle's Flushing medium and then spun in the same manner as the semen samples.

4.4 ISolate® Discontinuous Gradients

4.4.1 Preparation of ISolate® solutions

The ISolate® solutions were prepared as described in Table 4..3. Once prepared the solutions were stored at 4°C until used.

Table 4.3: ISolate® solutions

Solution (% of ISolate®)	ISolate® (ml)	Earle's Flushing Media (ml)
95	2.85	0.15
90	2.70	0.30
85	2.55	0.45
80	2.40	0.60
75	2.25	0.75
70	2.10	0.90
65	1.95	1.05
60	1.80	1.20
55	1.65	1.35
50	1.50	1.50
45	1.35	1.65
40	1.20	1.80
35	1.05	1.95
30	0.90	2.10

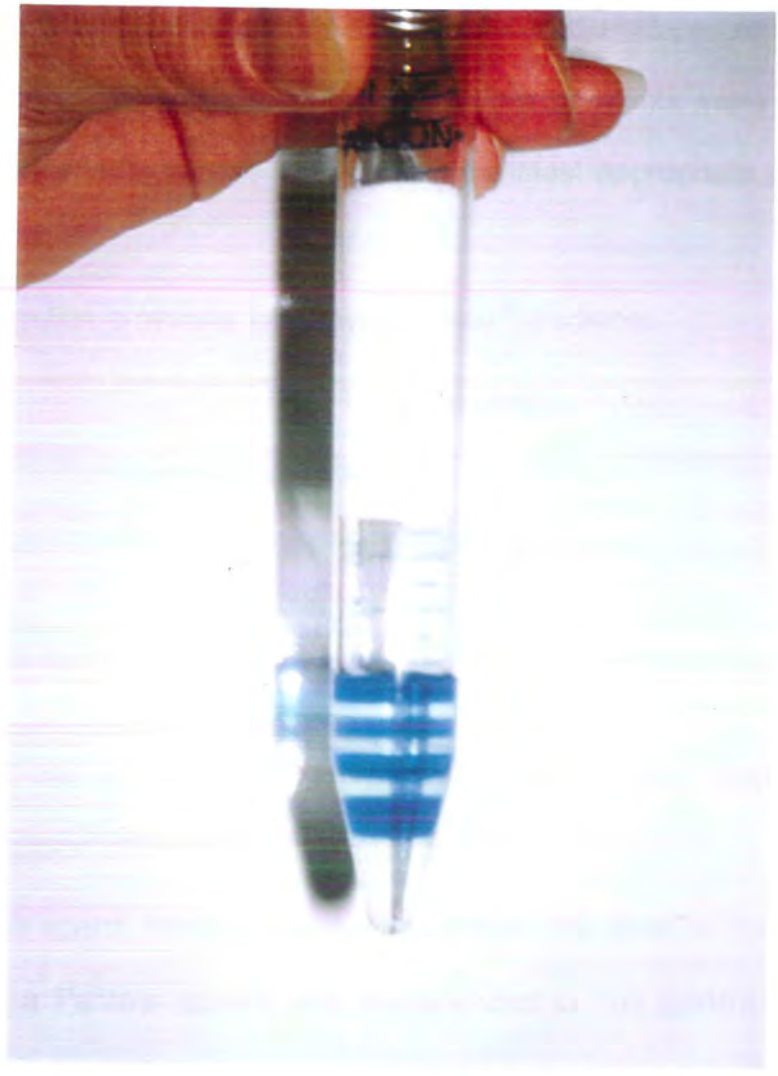
4.4.2 15-layered ISolate® Discontinuous Gradients

The ISolate® solutions described above were used to produce the gradients. A layer of 0.3ml 100% ISolate® was added to a sterile 15ml tissue culture tube using a Pasteur pipette. On top of this layer were placed layers of each of the ISolate® dilutions from 95% to 30%. Each of the layers consisted of 0.3ml of solution and they were layered using a Pasteur pipette ensuring that the interface between the layers was not disrupted.

4.4.3 8-layer ISolate® discontinuous gradients

For this gradient only the 100%, 90%, 80%, 70%, 60%, 50%, 40% and 30% ISolate® solutions were used. As in chapter 4.4.1, the gradients were formed in 15ml tissue culture tubes, with the 100% layer being placed at the bottom of the tube. Each of the layers consisted of 0.3ml of solution, and extreme care was needed to ensure that the sequential addition of each layer did not disturb the interfaces within the gradient. The gradients were formed immediately before use to avoid the possibility of degeneration.

Figure 4.5: 8-layer ISolate® discontinuous gradients



Once the gradient was formed, a sample of semen or washed semen, usually 1ml in volume was layered on top. The washed semen was produced as described in chapter 4.2.9. The volume of sample layered on to the gradient was dependent on the volume of the semen sample, where possible, 1ml of semen or washed semen was used, but with samples of very low volume it was necessary to use 0.5ml.

4.3.4 Centrifugation of ISolate® Discontinuous Gradients

Using the Hettich Zentrifugen Universal 16A centrifuge within the Andrology laboratory of Concept Fertility Centre, the gradients were initially tested four different speeds, in order to establish the most appropriate protocol for the study:

Table 4.4: Centrifugation protocols for 8-layer ISolate® gradients

Speed (g)	Time (minutes)
110	30
220	25
360	20

It was shown that the 110g and 220g protocols were the most appropriate speeds for centrifugation of the ISolate® gradients.

The required sperm fraction was removed from the base of the tissue culture tube using a Pasteur pipette and resuspended in 1ml Earle's Culture Medium. The fraction was then washed as described in chapter 4.3.10, before being resuspended in Earle's Culture Medium containing 10% human serum to produce a final volume of 1ml.

The total and motile counts and sperm progression of the final fractions were determined using the same hemocytometer as used for the semen analysis, and in the same manner as described in chapter 4.3.6 and 4.3.7. It should be noted that in this case the correction factor to determine the total and motile counts in millions/ml differs from that for the semen dilution, see Table

4.2. 0.5ml of the final fraction was then kept for the survival study whilst the other half of the sample was prepared for FISH, see chapter 4.6.2.

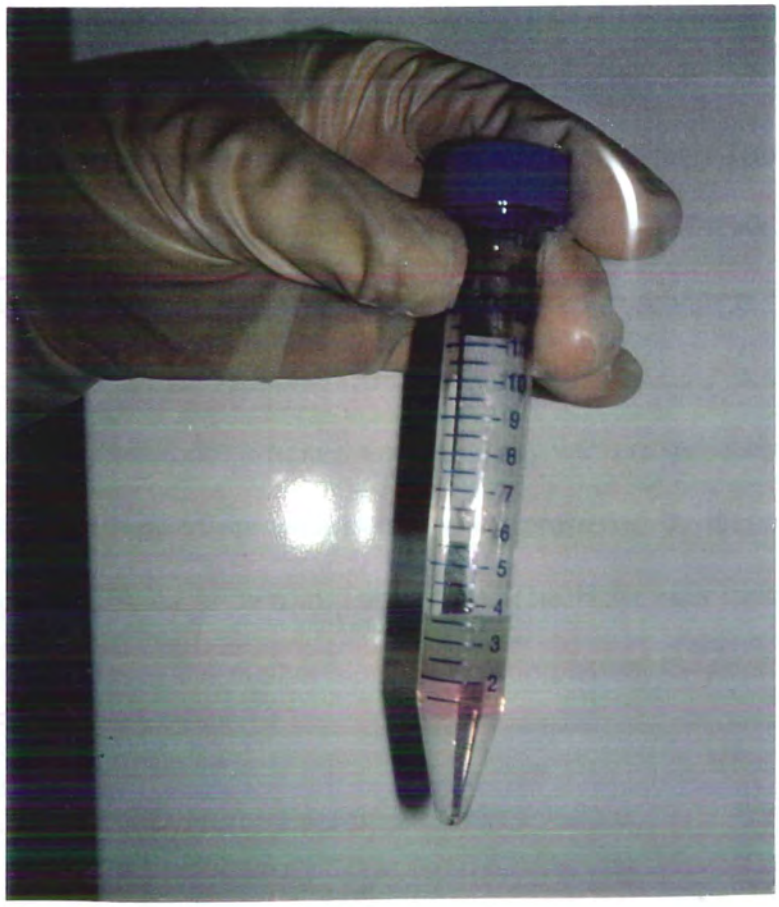
To determine if there were significant differences in the percentage recoveries and percentage motile sperm between gradients, multiple identical gradients were run using the same samples.

4.3.5 Sperm Survival following 8-layer ISolate® Discontinuous Gradients

To determine if either X- or Y- bearing sperm exhibit greater survival, the following protocol was applied to 0.5ml of the final ISolate® factions. The fractions were stored at room temperature (21°C - 26°C), out of direct sunlight in the Andrology laboratory at Concept Fertility Centre. The total and motile counts of the sample were determined every 24 hours until the motile count was 50% of that measured immediately after being run through the 8-layer ISolate® discontinuous gradient. The total and motile counts were determined in the same manner as in chapter 4.3.6.

Once the motile count reached 50% of the initial value, the motile sperm was separated using a 2-layered ISolate® Gradient. 1ml of 90% ISolate® solution (0.9ml ISolate® and 0.1ml Earle's Flushing Medium) was placed in a 15ml tissue culture tube and 1ml of 45% ISolate® solution (0.45ml ISolate® and 0.55ml Earle's Flushing Medium) was layered on top, using a Pasteur pipette to ensure the gradient interface was not disturbed. Finally, 0.5ml of the 8-layer ISolate® treatment sperm was layered on top and the gradient was centrifuged at 360g for 20 minutes. The resultant pellet was washed as described in chapter 4.3.9 and prepared for FISH analysis.

Figure 4.6: 2-layer ISolate® gradient



4.5 Human Serum Albumin Columns

4.5.1. Preparation of Human Serum Albumin Gradients

The gradients were formed in heat-sealed Pasteur pipettes. The pipettes were broken at the point of tapering, and the end sealed by placing it into a Bunsen burner flame. The pipettes were usually prepared in advance to ensure that they were cool before being used. Prior to use, the sealed pipettes were checked to ensure they were not cracked and that they were completely sealed.

The solutions of human serum albumin were prepared by dissolving the required amounts of HSA as shown in Table 4.5. The HSA was dissolved by placing the tubes containing the suspension on a mixing wheel for approximately 2 hours or until dissolved.

Table 4.5: Human serum albumin solutions

Solution (%)	Human serum albumin (g)	Earle's Flushing Media (ml)
20	1.0	5
15	0.75	5
10	0.5	5

0.3ml of the 20% solution was placed at the base of the gradient column using a Pasteur pipette. The 15% and then 10% layers were placed on top, ensuring that the interfaces between the layers were not disturbed as shown in figure 4.7.

Figure 4.7: Human serum albumin gradient

4.5.2 Use of Neat Semen with Human Serum Albumin Columns

Initially the columns were layered with 0.3ml of neat semen and then kept at room temperature for a set length of time, of 60, 45, 30, 15 or 10 minutes. After this period, the semen layer was removed, the columns were left for a second, equal length of time. The 10% and 15% layers were then removed, while the 20% fraction was washed as described in chapter 4.3.10, and prepared for fluorescence *in situ* hybridisation. Multiple columns were run using the same sample, to determine if there was any significant variation between the columns.

4.5.3 Use of 2-layer ISolate® treated sperm

Due to problems encountered with using neat semen, the semen was pre-treated using a 2 layer ISolate® gradient. The two ISolate® layers consisted of 90% and 45% ISolate® (see chapter 4.4.5 for more details), as depicted in figure 4.5.

Both the 90% and 45% layers were 1ml in volume and they were layered using a Pasteur pipette in the same manner as the 8 layer ISolate® gradients. Usually 1ml of the neat semen was then layered onto the gradient as shown above, this volume was however dependent upon the size of the sample. The gradients were centrifuged in the Hettich Zentrifugen Universal 16A centrifuge in the Andrology laboratory at Concept at 360g for 20 minutes. The resulting pellet was then washed and resuspended in 1ml of Earle's Culture Medium as usual. The processed sperm count and motility were accessed as described in chapter 4.3.6.

0.5ml of the 2-layer ISolate® treated sperm was prepared for fluorescence *in situ* hybridisation, whilst the second 0.5ml was layered on to a human serum albumin gradient as for the neat semen. Due to it being suggested that the ideal percentile recovery of motile sperm from the human serum albumin gradients being less than 10%, (Pyrazak, 1994). the time that the sperm sample was on the gradient had to be reduced. For the same reason the time expended in its removal of the 10% and 15% human serum albumin layers also had to be reduced (see chapter 3.2). It was shown that by removing the sperm layer after 7.5 minutes and then removing the 10% and 15% layers after a second lapse of 7.5 minutes was the most reliable in achieving final fractions

of sperm which had a motile count of over 90% and had a motile recovery which was less than 10% of the initial motile count.

4.6 Fluorescence *in situ* hybridisation

4.6.1 Preparation of standard cell suspensions

The standard cell suspensions used were derived from samples that had already been processed by the Cytogenetics Department of King Edward Memorial Hospital. The samples had been prepared using the following method:

Approximately 2ml of peripheral blood, which had been collected with sodium heparin, was used to set up two synchronised cultures. Into two sterile 10ml culture tubes was placed 0.25ml of whole blood and 5ml of Opti-MEM supplemented medium. With the cap loosely in place, the tubes were incubated at 37°C in a CO_2 enriched incubator for 48 hours.

After this time the tubes were removed from the incubator and 100µl of thymidine was added to block DNA synthesis, the tubes were then returned to the incubator for approximately 17 hours. 100µl of deoxycytidine was added to the tubes to release the DNA synthesis block and the cell culture was allowed to continue for 4 hours at 37°C. 50µl of colcemid was added to the tubes, and they were incubated for a final 30 minutes before harvesting of the cells.

The blood cell culture was transferred to centrifuge tubes and centrifuged at 350g for 5 minutes. The supernatant was removed, the pellets were resuspended in 5ml of 0.057M KCl and mixed well before being centrifuged

again at 350g for 5 minutes. Addition of the hypotonic KCl solution lysed the erythrocytes, and hence the supernatant had to be carefully decanted following centrifugation. 4% Acetic acid was used to resuspend the pellet and the suspension was centrifuged as before. The final pellets were then resuspended in 5ml of fresh fix (3 parts methanol to 1 part acetic acid) and again centrifuged. All but 0.2ml of the supernatant was removed and the cells resuspended to the required concentration, the suspension was used in the preparation of slides (see chapter 4.6.3).

4.6.2 Preparation of sperm suspensions

The sperm samples were transferred into a labelled 15ml centrifuge tube using a disposable pipette. The total volume was made up to 10ml using phosphate buffered saline (PBS), and the sample mixed well before being centrifuged for 5 minutes at 350g. The resultant supernatant was discarded and the pellet resuspended in PBS before being respun in the same manner.

A stock solution of fix (3:1 methanol:acetic acid) was prepared immediately prior to use. The sperm pellets were resuspended in 10ml of fix and left to stand for 10 minutes at room temperature (approximately 22°C). The suspension was then centrifuged for 5 minutes at 350g and the supernatant discarded. The pellet was then washed a further two times using 5ml of fix in the same manner as the PBS washes described above. The final sperm pellet was resuspended in 0.5ml of fresh fix.

4.6.3 Slide preparation

Using a thin tipped 1ml disposable pipette a drop of the required cell or sperm suspension was dropped on to the centre of a pre-cleaned slide from a height of approximately 5cm. Just before the cell suspension dried, a drop of fix was placed directly on top. The slides were dried in a 60° C oven for 10 minutes and then checked under a Leitz Laborlux D phase contrast microscope at 160x magnification to ensure that the concentration of sperm was suitable. If insufficient sperm were present, a second drop of the suspension was placed on the slide, which was dried and then re-examined. Conversely, if the sperm concentration was too high, the sperm suspension was diluted and a second slide prepared.

4.6.4 Storage of any unused sperm suspension

Any unused sperm suspension was stored at -20°C in fixative until the FISH result was available. Once a result was obtained the unused portion of the sperm suspension was discarded. If required, however, it would have been possible to store these sperm suspensions in an useable condition for testing for up to 12 months at -20°C.

4.6.5 Pre-treatment of cells

The slides were sequentially incubated in the following ice cold ethanol solutions for 2 minutes each, 70%. 95% and 100%. After draining, the slides were dried on a heating block at 70°C. A drop of the denaturation mix (see Appendix G) was placed on to the slides and they were again covered with a

plastic coverslip. After two minutes the slides were removed from the block and they were immediately immersed into pre-chilled 2xSSC (see Appendix G) for 5 minutes. Finally, the slides were reincubated in the ice cold 70%, 95% and 100% ethanol solutions again for 2 minutes each (Cytogenetics Department KEMH, no date)

4.6.6 Trypsin/DDT decondensation of sperm

For this protocol it was necessary to let the slides completely dry and fix to the slide overnight. 40µl of Trypsin/DTT (see Appendix G) was pipetted directly on to the sperm slides, they were then covered using a plastic coverslip and incubated at 25°C for 20 minutes. The slides were washed twice with PBS for 5 minutes at 25°C, and briefly allowed to drain and air dry before being sequentially incubated in the following ice cold ethanol solutions for 2 minutes each, 70%, 95% and 100%.

The slides were then drained and dried by placing on a heating block at 70°C. A drop of the denaturation mix (see Appendix G) was placed on to the slides and they were again covered with a plastic coverslip. After two minutes the slides were removed from the block and they were immediately immersed into ice-cold 2xSSC (see Appendix G) for 5 minutes. Finally, the slides were reincubated in the ice cold 70%, 95% and 100% ethanol solutions again for 2 minutes each (Cytogenetics Department KEMH, no date)

Before hybridisation the slides were examined under the Leitz Laborlux D phase contrast microscope at 160x magnification to ensure that the sperm

heads had been decondensed but not over-digested. If the sperm had not been decondensed or had been over-digested a second slide was prepared.

4.6.7 Sodium hydroxide decondensation of sperm

Immediately after preparation (chapter 4.6.3) the slides were baked in a 60°C oven for 15 minutes and then allowed to cool for 10 minutes at room temperature (approximately 22°C). A drop of 3M sodium hydroxide was placed on to the slides and they were then covered with a plastic coverslip. After incubating at room temperature (approximately 22°C) for 3 minutes, the coverslips were removed and the slides washed for 30 seconds first in PBS, then in distilled water and finally in fresh fix (see chapter 4.6.2). The slides were dried in a 60°C oven for 5 minutes and then examined under a Leitz Laborlux D phase contrast microscope at 160x magnification.

If the majority of sperm heads were not decondensed the slide was treated with sodium hydroxide for a further minute and then re-examined. Once the sperm were decondensed the slides were sequentially incubated in 70%, 95% and 100% ice cold ethanol solutions for 2 minutes. The slides were then allowed to drain and air dried.

4.6.8 Hybridisation of chromosomal probes

Oncor (Gaithersburg, MD, USA) supplied the probes used and the accompanying recommended protocol was followed. The X chromosomal probe was DXZ1, which is an alpha satellite probe that specifically binds to highly repetitive DNA at the centromere of the human X chromosome. The Y

chromosomal probe, DYZ1, is a classical satellite probe, which hybridises to the short, repeats in the satellites located in the pericentric heterochromatin of the human Y chromosome.

12μl of the Probe Mix (see Appendix G) was pipetted on to each slide. The slides were covered with a glass coverslip, the edges sealed with rubber cement, and they were incubated in a humidified box at 37°C overnight (for a minimum of 18 hours).

4.6.9 Detection of chromosomal probes

During the whole of the detection procedure there is the need to ensure that the slides are not allowed to dry out completely. The rubber cement and coverslips were carefully removed from the slides to ensure that the cells were not scrapped off. The slides were washed three times for 5 minutes each in 2xSSC/50% FA at 43°C with gentle shaking (see Appendix 6). The slides were briefly drained and then washed for 10 minutes in 0.1xSSC with shaking (see Appendix 6) at 43°C. 50μl of 4xSSC/1% BSA (see Appendix 6) was pipetted on to the slides, which were covered with a plastic coverslip and incubated at 25°C for 15 minutes. After which the slides were briefly drained of the 4xSSC/1% BSA.

20μl of Detection solution 1 (see Appendix G) was pipetted on to each slide. The slides were covered with a plastic coverslip and they were incubated at 25°C for 15 minutes. The slides were then washed twice with shaking at room temperature in 4xSSC/0.05%TW20 (see Appendix G) for 5 minutes and briefly drained. 20μl of Detection solution 2 (see Appendix G) was pipetted on

and again the slides were covered and incubated for 15 minutes. They were next washed in 4xSSC/0.05%TW20 and 20µl of Detection solution 3 (see Appendix G) was added by pipette. The slides were incubated and washed as for the previous two detection solutions, then washed for 5 minutes at room temperature in 1xPBS with shaking. After brief draining and air drying, 10µl of DAPI-Antifade (see Appendix G) was added by pipette, the preparations were covered with a glass coverslip and the edges sealed using clear nail varnish.

4.6.10 Fluorescence microscopy

The slides were observed under the Ortholux Ploemopak 2 fluorescence microscope on the day of the detection procedure. The Leica filters used are described in the Table 4.6.

Table 4.6: Filters used for fluorescence microscopy

Leica Filter block	Excitation range	Exciting filter	Used to observe
A	U.V.	Band pass filter 340-380nm	DAPI
I2	Blue	Band pass filter 450-490nm	FITC
N2.1	Green	Band pass filter 515-560nm	Rhodamine

4.6.11 Sperm counting criteria

Using the Ortholux Ploemopak 2 fluorescence microscope, it was necessary to change the filters to permit observation of the probes and the background DAPI stain. For this reason, it was important to ensure that the

signal observed was not only located within the sperm head but also did not result from random binding. The following criteria, adapted from Bibbins et al (1988), were applied when counting and only those sperm heads, which meet the criteria, were included.

1. The sperm must be of normal size and shape.
2. The individual sperm heads must be identifiable.
3. The sperm head membranes must be intact.
4. The signal must be within the sperm head.
5. It must be possible to focus the signal into a distinct point, diffuse signals are usually background.

Using these criteria 500 sperm heads were usually counted. There was the need to count this number of sperm because Moore and Gledhill (1988) determined that this was the minimum number of sperm that would statistically show a change in the sex ratio. A note was kept if the percentage of unlabelled sperm seemed to be high, this was an indication that the sperm heads had not been sufficiently decondensed. There was also the possibility that by having a high number of unlabelled sperm the ratio of X- to Y- bearing sperm counted was not a true representation of the sample. When it was not possible to count 500 sperm, as many as possible were counted.

The Orthomat Microscope Camera with Kodak® Ektochrome slide film was routinely employed to take photographs of the standard cells and the sperm.

5. Results

5.1 Establishing FISH techniques

5.1.1 Standard cell spreads

To confirm that the DXZ1 and DYZ1 probes hybridised to the X and Y-chromosomes respectively, chromosome metaphases were tested according to the prescribed protocols. The metaphase spreads were from samples containing male (46,XY), female (46,XX), Klinefelter syndrome (47,XXY) and a mosaic of Turner syndrome (45,X;46,XX) cells. The samples were prepared as described in chapter 4.5.1. This methodology is designed to produce metaphase preparations, however it is usual for the suspensions produced to contain both meta- and interphases.

For the 46,XY, 46,XX and Klinefelter samples two slides were prepared and processed by FISH, while for the Turner sample one slide only was prepared. The Turner sample used was a mosaic and it was found not to contain metaphases. Therefore it was not possible to deduce which of the interphases contained only one signal, as the probe could not bind to the second X chromosome. Several metaphases and interphases were examined on each slide to verify that the probes had in fact bound correctly to the chromosomes. If no metaphases were visible, as was the case with the 45,X;46XX sample, only interphases were examined.

In the set-up arrangement of the microscope used to examine the preparations, different filters were required for observing and photographing samples (for details see chapters 4.5.10 and 4.5.11). To enhance the photographic record, three images were superimposed using the computer program Microsoft Photo Editor. Examples of the interphases and metaphases

from the samples prepared are reproduced in Figures 5.1 to 5.4. The examples provided indicate the difference between the photographs as originally taken and those produced by the computer-based image superimposition technique.

Figure 5.1: Superimposed metaphase spread of a female cell
The chromosomes are stained blue due to the DAPI stain, the two red dots are the two rhodamine stained X chromosome centromeres.

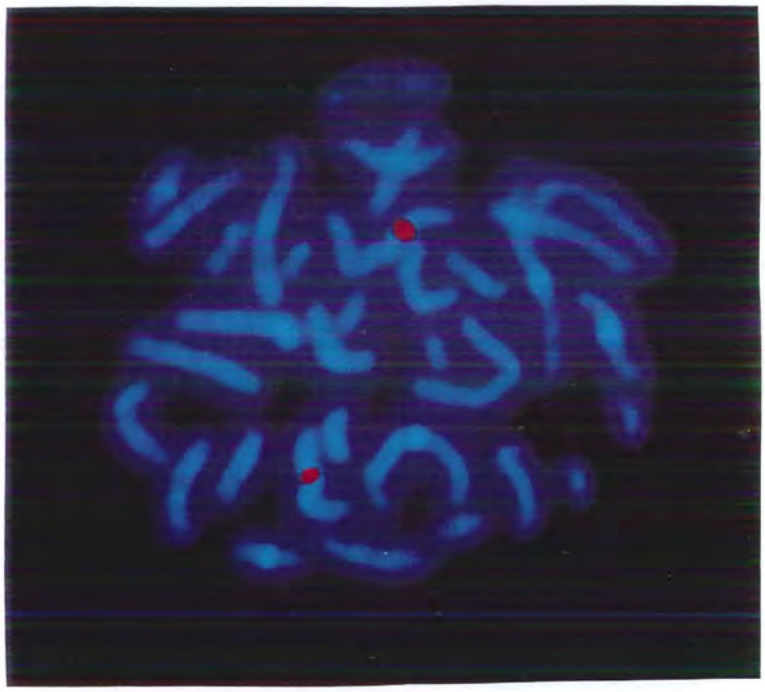


Figure 5.2: DAPI stained female interphase cell

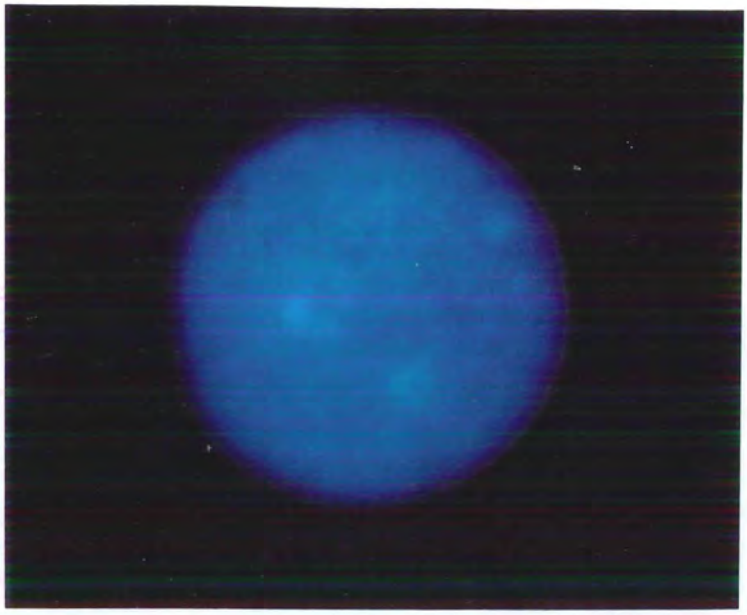


Figure 5.3: Rhodamine stained DXZ1 labelled female interphase cell

This is the same cell as shown above, only viewed using a different filter (see chapter 4.4.10), the two bright red dots seen are the rhodamine stained centromes of the two X chromosomes.

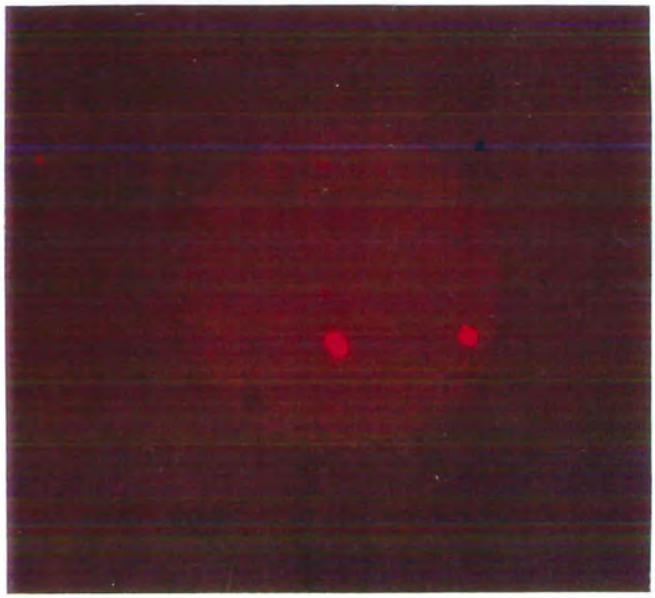
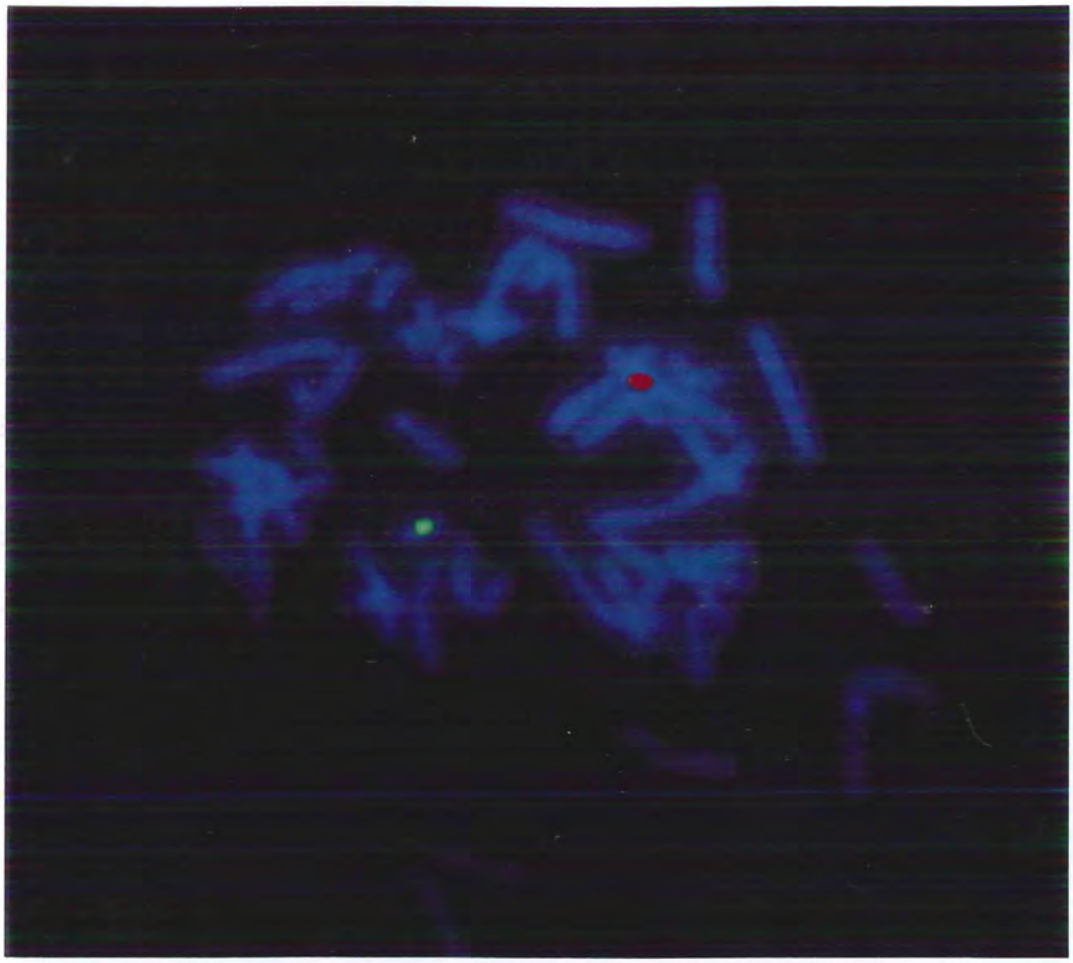


Figure 5.4: Superimposed metaphase spread of a male cell

All of the chromosomes are stained blue due to the DAPI, the red dot indicates the X- chromosome due to the rhodamine staining of the centromere, whilst the green dot locates the Y- chromosome because of the fluorescein staining of the pericentric heterochromatin.

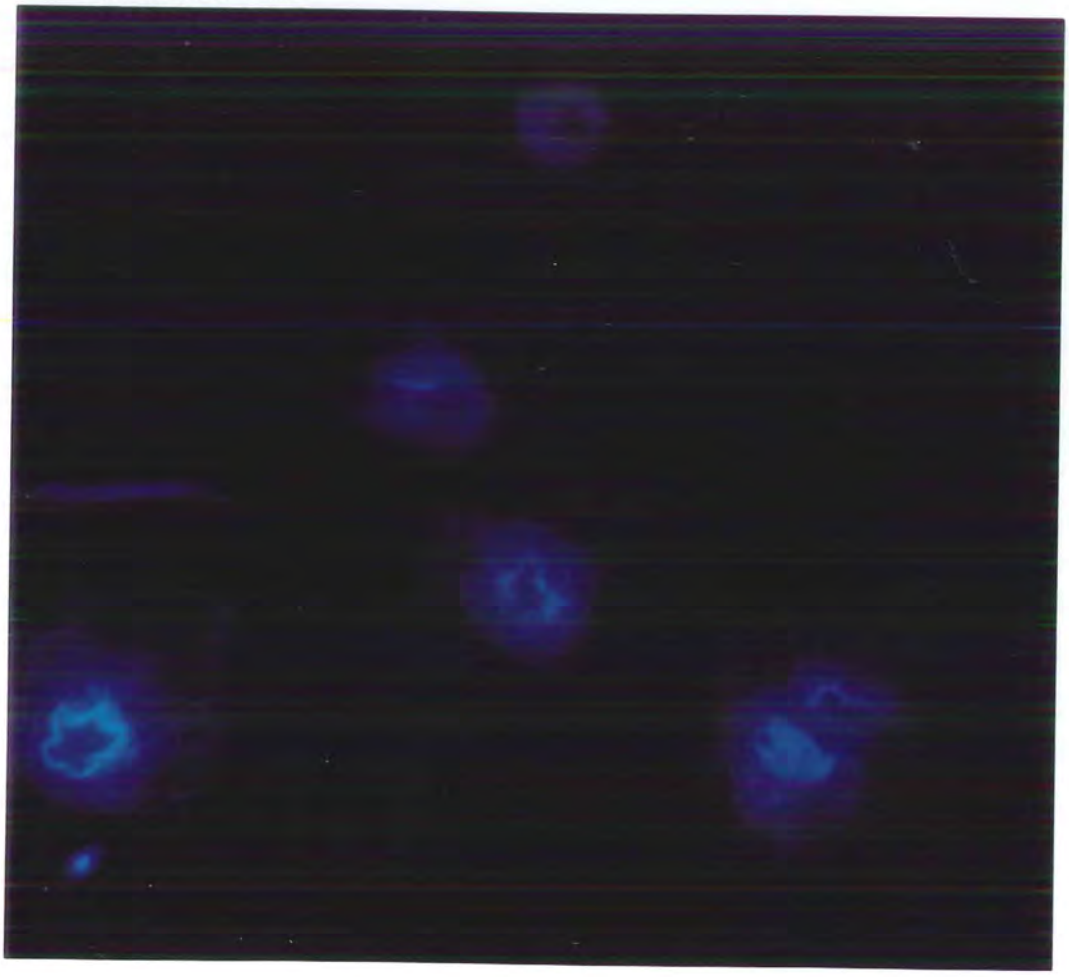


5.1.2 Trypsin/DTT pre-treatment of sperm

As discussed in chapter 3.4, sperm nuclei must undergo decondensation before hybridisation can occur. As a Trypsin/DTT method was in routine use within the Cytogenetics Department, KEMH, this technique was initially adopted. However, a number of problems were encountered which resulted in sperm heads either being over-digested or not decondensed. For example, Figure 5.5 shows sperm heads that have been over-digested.

Figure 5.5: DAPI stained over-digested sperm heads

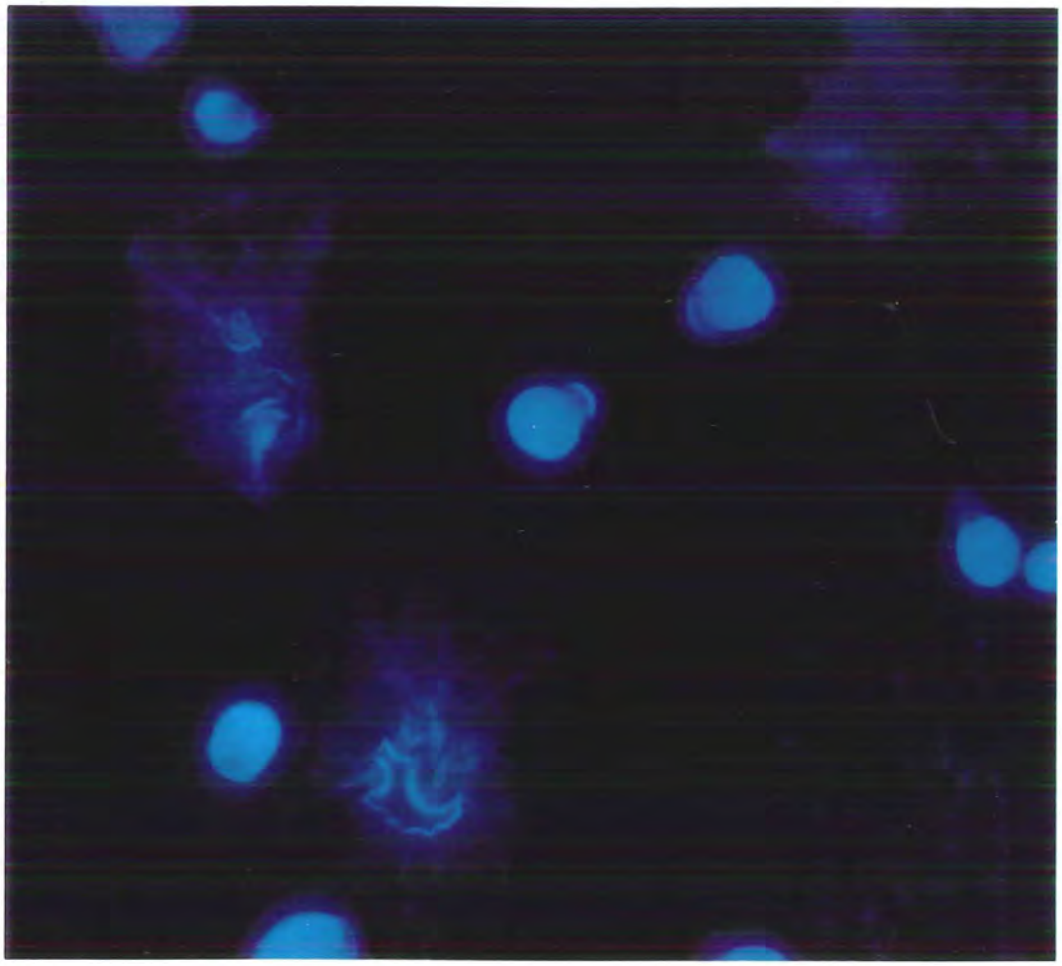
The DAPI stained sperm heads all appear shriveled, which indicates that the cells have been over-digested and hence the cellular membrane has broken down.



As well as the sperm heads on individual slides either being over-digested or not decondensed, there was a problem with some sperm heads on a single slide being over-digested whilst others remained non-decondensed. An example of this type of problem can be seen in Figure 5.6.

Figure 5.6: DAPI stained trypsin pre-treated sperm heads

As can be seen some of the sperm heads are shriveled and therefore have been over-digested, others are still small and compact, thus indicating that they are not decondensed sufficiently.



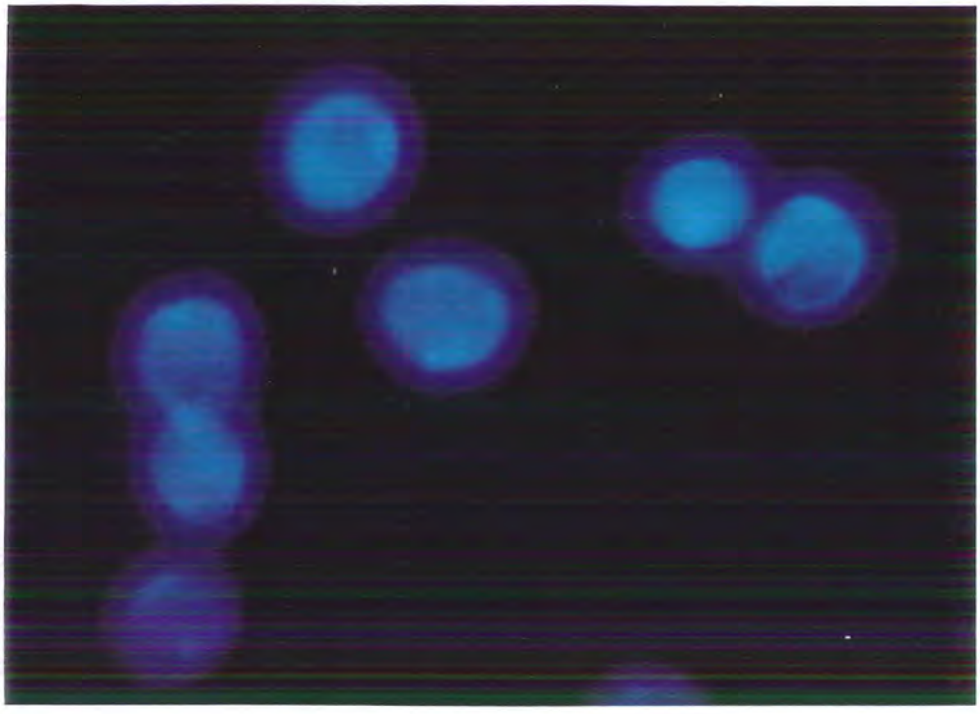
In an attempt to avoid this difficulty, the temperature at which the pre-treatment was conducted was altered. By varying the temperature through a range of 20°C – 30°C, it was determined that the process was very temperature-sensitive. The room temperature of the laboratory was monitored and a temperature variation of between 19°C and 29°C recorded. However, after testing a number of different protocols, it was not possible to establish conditions where the majority of sperm heads would be decondensed because of inter-sample variation. As a result of these problems, it was decided that an alternative method of pre-treating the sperm was needed.

5.1.3 Sodium hydroxide pre-treatment of sperm

This pre-treatment technique provided a more constant method for the decondensation of sperm heads than the Trypsin/DTT protocol, as illustrated in Figure 5.7.

Figure 5.7: Sodium hydroxide pre-treated sperm heads

All of the sperm heads appear swollen indicating that they have been decondensed sufficiently to allow probe entry and binding.



The efficiency of sodium hydroxide processing is dependent on the ambient room temperature and the age of the sperm spread. In an attempt to avoid problems with over-digestion and non-decondensation of sperm heads, a test slide was prepared each time a set of slides was produced. The test slide was treated with sodium hydroxide for 3 minutes and then examined using light microscopy. If the majority of sperm heads had not decondensed the slide was treated with sodium hydroxide for a further 30 seconds and then re-examined. This process of examination and re-treatment was continued until the majority of sperm heads in the sample had decondensed. The time required to suitably

decondense the sperm heads on the test slide was then used for the sodium hydroxide treatment of the remaining slides. However, it was still necessary to check each slide individually and possibly re-treat with sodium hydroxide for longer, or even prepare a second slide, because the sperm heads had been over-digested.

On average, the sperm processed using human serum albumin required an additional 30 seconds of sodium hydroxide treatment to ensure decondensation, by comparison with sperm samples prepared either by washing using medium or ISolate®.

5.1.4 Counting X and Y probe signals

To determine whether the signals counted were being accurately assessed, an experienced FISH technician (JP) independently recounted 10 slides previously prepared using the Trypsin/DTT pre-treatment method during the initial studies. The counting criteria used were as described in chapter 4.6.11. Table 5.1 gives a comparison of the percentage of X- and Y- bearing sperm counted (mean ± standard error) by JP and myself (JI) in the 10 slides tested.

Table 5.1: Comparison of percentage of X- and Y- bearing sperm counts between JI and JP (group 1)

	JI % X- bearing sperm	JP % X- bearing sperm	JI % Y- bearing sperm	JP % Y- bearing sperm
Mean	53.4	50.2	46.1	49.8
SE	1.61	1.47	1.49	1.46

Although there was a correlation between the two sets of counts, the difference was statistically significant for both the X and Y counts:

X- bearing sperm: $t = 2.312$, $df = 9$; $p = 0.046$

Y- bearing sperm: $t = -2.783$, $df = 9$; $p = 0.021$

Because of the significant difference between the counts obtained, a second group of slides (n=7) were randomly selected and again counted independently by myself (JI) and JP. This group of slides were all pre-treated using sodium hydroxide.

Table 5.2: Comparison of percentage of X- and Y- bearing sperm counts between JI and JP (group 2)

	JI % X- bearing sperm	JP % X- bearing sperm	JI % Y- bearing sperm	JP % Y- bearing sperm
Mean	51.0	51.0	49.0	49.0
SE	0.98	1.29	0.98	1.29

With this second test protocol, there was no significant difference between the two sets of counts.

X- bearing sperm: $t = -0.04$, $df = 6$; $p = 0.970$

Y- bearing sperm: $t = 0.04$, $df = 6$; $p = 0.970$

All of the slides used in this second group had hydridisation efficiencies of at least 90%, this was an improvement over the first group of slides which had been pre-treated using Trypsin/DTT and had hydridisation effciences of approximately 60%.

5.2 Semen analysis: initial studies to establish reproducible techniques

Considerable time was invested to ensure that the total and motile sperm counts were not only reproducible but also comparable with those reported by an experienced andrology technician (MG). For the test, 10 sperm samples were randomly selected and were independently counted by JI and MG, using both a Neubauer Improved Chamber and a Makler chamber. Use of both chambers additionally permitted a comparison to be drawn between the two methods (see chapter 4.2.5). Table 5.3 shows the comparison of the counts (n=10) obtained by JI and MG using a paired samples t test.

Table 5.3: Comparison of sperm counts by JI and MG

Chamber	Type of Count	JI Mean (millions/ml) [SE]	MG Mean (millions/ml) [SE]
Neubauer	Motile	4.70 [0.92]^{A}	4.46 [0.74]^{A}
Neubauer	Total	5.39 [0.93]^{B}	5.00 [0.79]^{B}
Makler	Motile	7.73 [2.20]^{C}	7.80 [1.60]^{C}
Malker	Total	10.44 [2.28]^{D}	10.15 [1.57]^{D}

A: $t = 0.91$, $df = 9$; $p = 0.39$ B: $t = 1.28$, $df = 9$; $p = 0.23$

C: $t = -0.05$, $df = 9$; $p = 0.96$ D: $t = 0.21$, $df = 9$; $p = 0.84$

There was no statistically significant difference between the counts obtained by JI and MG in any of the four categories tested. In order to determine if there was any difference between the counts obtained with the two types of chamber, the total and motile Neubauer and Makler counts were compared using a paired samples t test.

Table 5.4: Comparison of sperm counting chambers

Operator	Comparison of counts	Result
JI	Motile counts from Neubauer and Makler chambers	t = -1.84, df = 9; p = 0.10
JI	Total counts from Neubauer and Malker chambers	t = -2.92, df = 9; p = 0.02
MG	Motile counts from Neubauer and Malker chambers	t = -3.15, df = 9; p = 0.01
MG	Total counts from Neubauer and Malker chambers	t = -5.55, df = 9; p = 0.001

In Table 5.4 it can be seen that there was a significant difference between the total counts by JI and the total and motile counts by MG using both types of chamber. As a result of these differences, and the suggestion by Mortimer (1990) that the Makler sperm counting chamber is not as accurate as the Neubauer Improved Counting chamber, the Neubauer Chamber was subsequently used for all of the counts in both the pilot and clinical studies.

5.3 Establishing ISolate® techniques

5.3.1 15 layer discontinuous ISolate® gradients

Initially a 15 layer discontinuous ISolate® gradient was planned, however a number of problems were encountered with the formation of the gradients. As explained in chapter 4.4.2, the 15 layer gradients were to be formed by sequentially layering the various ISolate® solutions on top of each other, while ensuring that the interfaces between the layers were not disturbed. In practice

this proved to be very difficult and time-consuming, usually because the application of a new layer disturbed an interface already formed within the gradient or the gradient had undergone some degree of degeneration.

The likelihood of disturbing interfaces within the gradient by adding new layers of solution became greater as the number of layers progressively increased. This was probably due in part to the V-shape of the centrifuge tube used. Thus, as additional layers were added, they decreased in depth and were less stable. Degeneration of the gradients probably arose because of the small concentration gradients between the sequential ISolate® solutions used. For both of these reasons, it was decided that an ISolate® gradient consisting of fewer layers would be preferable and more practical. It is however possible that a satisfactory 15-layer gradient could be achieved using a different medium, as at least 12-layer gradients have been formed using Percoll (see chapter 3.3).

5.3.2 8 layer discontinuous ISolate® gradients

The formation of these gradients proved to be more reliable than the 15 layer gradients. It was possible to form the gradients up to an hour in advance, and as long as the gradients remained undisturbed no degeneration occurred. However, the gradients had to be checked carefully to ensure that the interfaces remained intact before application of the semen sample. This was important because, unlike the gradient shown in Figure 4.5, the solutions used did not incorporate a stain to enhance visualisation, and there was almost no difference in the colours of the different layers.

Once the formation of the gradients had been established a suitable speed and time of centrifugation had to be determined. As stated in chapter 3.2, it was desirable that the percentage recovery of motile sperm was less than 10% of the initial motile count. The first protocol tested was based on the centrifugation speed and timing in 2-layer ISolate® gradients used within the Andrology laboratory of Concept Fertility Centre for washing semen samples, that is 360g for 20 minutes. Both washed and neat semen were treated using the 8 layer ISolate® gradients to determine if any benefit was obtained by washing semen samples before application on to the gradient. In a number of published methods for the enriching X- or Y- bearing sperm, the semen sample was washed and diluted to a standard concentration prior to the enrichment protocol. In the present study, the semen samples that were washed before application on the gradient were not diluted to a standard concentration, as the percentage motile recovery could be determined without the need for a standardised concentration.

Tables 5.5 and 5.6 summarise the results, means and standard errors, from the initial evaluation of the 8-layer ISolate® gradients layered with neat semen and with washed semen samples.

Table 5.5: Summary of results of washed samples

Centrifugal force (g)	Centrifugation (minutes)	n	Mean Recovery (%) [SE]	Mean Motility (%) [SE]
360	20	12	12.14 [2.36]	41.92 [5.83]
220	20	6	4.05 [2.45]	45.83 [20.83]
110	30	6	3.25 [2.04]	48.83 [18.30]

Table 5.6: Summary of results of neat semen samples

Centrifugal force (g)	Centrifugation (minutes)	n	Mean recovery (%) [SE]	Mean motility (%) [SE]
220	20	13	13.06 [3.08]	89.64 [3.57]
110	30	12	9.98 [3.42]	80.58 [7.26]

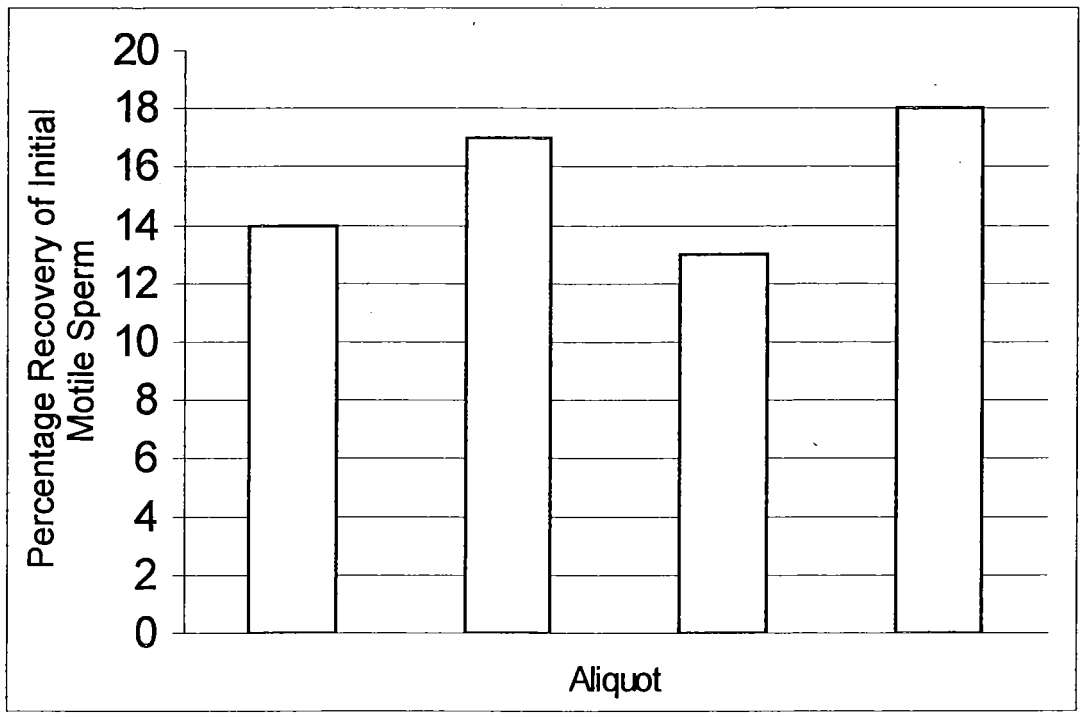
On the basis of these results, it was decided that there was no benefit in washing the semen sample prior to application on to the gradient. As both of the protocols for the neat semen samples gave appropriate results, with higher mean motilities than the washed samples, these methods were tested during the pilot study.

5.3.3 Reproducibility of 8-layer ISolate® gradients

To determine if the results obtained with individual discontinuous gradients were reproducible, several neat semen samples were divided into 4 aliquots and processed in an identical manner. The results are reproduced in

Figure 5.8. No significant difference was observed between the four aliquots in terms of either sperm recovery or sperm motility. A similar level of variability, again with no statistically significant difference between aliquots, was also observed when using washed semen, thus confirming that no benefit accrued from washing the semen prior to its application on the ISolate® gradient.

Figure 5.8: Determination of reproducibility of ISolate® gradients



5.3.4 The culture of ISolate® treated sperm

As previously discussed (see chapter 2.4) it was suggested by Shettles and Rorvik (1984) that X- bearing sperm might have a longer life span than Y-bearing sperm. To determine whether this was the case, the final ISolate®

treated sperm fractions were cultured in Earle's Culture Medium (see Appendix F) containing 10% pooled human serum, at room temperature (20°C-26°C). The samples were maintained until the number of motile sperm had been reduced to 50% of those present immediately after processing. The mean time required for culture was 40 hours (range 24 – 72 hours; n = 12). As it was not possible to determine a set period of time after which the number of motile sperm in all samples had been reduced to approximately 50% of the initial totals, each of the samples had to be individually monitored.

5.4 Establishing the human serum albumin column technique

As stated in the methods chapter (4.4.1), the HSA columns were formed in heat-sealed pasteur pipettes. Contrary to the experience with the ISolate® discontinuous gradients, no technical problems were encountered in the preparation of these columns. Table 5.7 summarises the results from the initial studies using HSA columns (mean and standard error).

Table 5.7: Neat semen layered on to columns

Time (minutes)	n	Mean recovery (%) [SE]	Mean motility (%) [SE]
60	6	23.17 [3.34]	71.67 [6.24]
45	5	20.80 [3.47]	72.80 [8.87]
30	8	15.00 [3.55]	64.00 [6.74]
20	5	3.02 [0.53]	79.20 [6.76]
15	6	14.50 [4.61]	62.17 [9.91]
10	2	22.00 [1.00]	65.50 [2.50]

As noted in chapter 3.2, an ideal final fraction would contain fewer than 10% of the initial motile sperm and they would be at least 90% motile. As can be seen in Table 5.7, none of the experimental conditions which were tested produced final fractions that consistently fulfilled these criteria. The main problem experienced was in producing samples with levels of motility greater than 90%. Within the Andrology laboratory of Concept Fertility Centre, a 2-layer ISolate® gradient consisting of 90% and 45% ISolate® layers is used to wash semen samples to produce final fractions which contain a high percentage of motile sperm. Therefore, in an attempt to increase the percentage of motile sperm in the final fraction, the semen was pre-treated using a 2-layer ISolate® gradient.

Table 5.8: HSA results using ISolate® Pre-treated semen

Time (minutes)	n	Mean recovery (%) [SE]	Mean motility (%) [SE]
15	3	19.00 [6.81]	94.67 [5.33]
7.5	7	7.74 [1.91]	88.80 [5.05]

Table 5.8 shows that after pre-treating the semen sample with a 2-layer ISolate® gradient the final fraction had the requisite high percentage of motile sperm. However, it next had to be determined whether the 2-layer gradient altered the ratio of X- to Y- bearing sperm, which in turn necessitated the processing of an aliquot of each of the 2-layer ISolate® pre-treated semen samples by FISH.

5.5 Pilot study

The aim of the pilot study was to ensure that the methodologies established during the initial investigations produced final fractions with recoveries of fewer than 10% of the initial motile sperm, and these fractions contained a high proportion of motile sperm (over 90%). The pilot study was also designed to show that it was possible to successfully process a semen sample using one of the methods established in the initial study, and then to treat the final fraction and the required control aliquots using fluorescence *in situ* hybridisation.

For both the HSA column and ISolate® discontinuous gradient methods, 5 samples were processed and the percentage of X- and Y- bearing sperm in the final fraction and the control aliquots determined.

5.5.1 ISolate® discontinuous gradient protocol

When the two ISolate® protocols were compared there was no significant difference between the recoveries and motilities obtained at the two centrifugation speeds (Table 5.9), therefore it was decided that it was unnecessary to proceed with both protocols. The 110g method had a mean recovery which was less than 10%, whereas the 220g recovery was greater than 10%. For this reason the 110g methodology was selected as the more suitable, and it was routinely used for testing the clinical samples.

Table 5.9: Summary of recoveries and motilities of ISolate® pilot samples

Centrifugation speed (g)	n	Mean recovery (%) [SE]	Mean motility (%) [SE]
220	5	12.55 [3.30]^{A}	70.58 [7.78]^{A}
110	5	9.76 [1.59]^{B}	64.67 [8.99]^{B}

A: $t = -0.73$, $df = 11$; $p = 0.48$ B: $t = -0.71$, $df = 11$; $p = 0.50$

As shown in Table 5.10 there was, however, no significant enrichment of X- bearing sperm at any stage of the process. In addition Table 5.11 shows that

there appeared to be no observable benefit in terms of enrichment of either X- or Y- bearing sperm to having a recovery of less than 10%.

Table 5.10: Summary of FISH results from ISolate® pilot samples

Sample	n	Mean % of X sperm at 110g [SE]	Mean of X sperm at 220g [SE]
Neat semen	5	50.02 [1.58]^{A}	50.02 [1.58]^{B}
ISolate® final preparation	5	54.84 [2.84]^{A}	50.10 [2.25]^{B}
After preparation by culturing	5	51.28 [2.62]^{A}	51.12 [2.12]^{B}

A : df = 2, F = 0.87; p = 0.46 B : df = 2, F = 0.10; p = 0.91

(Hybridisation efficiencies of between 90 - 96%)

Table 5.11: Summary of percentage changes with ISolate® pilot samples

	Mean % change of X-bearing sperm between neat semen & ISolate® final preparation [SE]	Mean % change of X-bearing sperm between ISolate® final preparation & cultured samples [SE]
Samples centrifuged at 110g	5.78 [3.02]	-1.50 [3.71]
Samples centrifuged at 220g	0.08 [1.30]	3.08 [2.73]
Samples with recoveries of <10%	4.46 [3.57]	-0.45 [2.80]
Samples with recoveries of >10%	0.64 [1.16]	2.65 [2.69]

The pilot study provided some useful technical pointers on the processing of ISolate® treated sperm by FISH. The sperm samples which had been cultured required additional washes with PBS and also fixative. Even after these additional steps, the samples often showed some background staining due to contamination with components of the culture medium. The cultured ISolate® samples also had very low sperm counts (fewer than 1 million/ml), which meant that it often was necessary to layer several drops of the sperm suspensions on to the slide.

The need to layer the sperm suspension seemed to contribute to the problem of non-specific background staining, and the requirement for a sufficient number of sperm on the slide had to be balanced against the need for minimal background contamination. With two of the culture samples the number of sperm layered on to the slides had to be reduced to an absolute minimum because of background contamination, and so the number of sperm heads

counted was fewer than 500. Hence there could be some doubt as to the significance of the culture results.

5.5.2 Human serum albumin columns protocol

The Human serum albumin protocol involving the sperm being layered onto the HSA gradient for 7.5 minutes resulted in a mean sperm recovery of less than 10% and the associated sperm motility was greater than 90%, which as discussed in chapter 3.2 are the optimal conditions for Y- bearing sperm enrichment (see Table 5.12).

Table 5.12: Summary of recoveries and motilities of HSA pilot samples

Stage	n	Mean recovery (%) [SE]	Mean motility (%) [SE]
2-layer ISolate®	5	47.28 [13.80]	80.34 [6.64]
7.5 min HSA column	5	7.74 [1.91]	88.80 [5.05]

However, even with these optimal recoveries and motilities, no significant enrichment in either X- or Y- bearing sperm was observed (see Table 5.13). There also was no change in the percentage of X- bearing sperm at any stage of the protocol (see Table 5.14).

Table 5.13: Summary of FISH results from HSA pilot samples

Sample	n	Mean % of X bearing sperm
Neat semen	5	51.40 [0.51]^{A}
2-layer ISolate® pre-treated preparation	5	51.40 [2.27]^{A}
HSA final preparation	5	50.00 [2.51]^{A}

A: df = 2, F = 0.22; p = 0.81 (Hydrisation efficiency of between 91 - 95%)

Table 5.14: Summary of percentage changes with HSA protocol

	n	Mean % change of X- bearing sperm between neat semen & ISolate® pre-treatment preparation [SE]	Mean % change of X-bearing sperm between ISolate® pretreatment preparation & final HSA preparation [SE]
All samples	5	0.00 [2.43]	1.40 [2.09]
Samples with recoveries < 10%	5	-0.50 [3.07]	0.25 [2.25]

The methodology did result in samples with low sperm counts (usually fewer than 1 million/ml), however no problem was encountered with these samples when they were processed using FISH. An observation made was that the HSA-treated sperm samples required slightly longer pre-treatment with sodium hydroxide (usually between 20 and 30 seconds) than either the washed neat semen or the ISolate® pretreated samples. This observation was not further investigated as the total time required for decondensing the sperm heads

varied between 3 minutes and 4.5 minutes, being dependent both on the sample and the temperature of the laboratory.

5.6 Clinical subjects

5.6.1 Summary of enquires received

Following the article on the project which appeared in The West Australian, and the subsequent publicity involving both local radio and television, a large number of enquires from potential donors were received at Concept Fertility Centre. The nature of the enquires received can be summarised as:

- Approximately 50 telephone calls received.
- The majority of calls were from married males, although some stated that their wife was the driving force behind the enquiry.
- Of those callers who provided details of their children, approximately 70% had two or more sons.
- 4 telephone calls were received from females who thought that sex selection treatments were available and wanted insemination.
- 1 call was from a man with no children, but who wanted to join the study.
- 1 letter was received from a man containing suggestions as to other possible methods of sex preselection.

From the initial enquires received, 27 men decided to join the study. The subjects had a median age of 36 years (range:31- 57 years) and 20 (74%) had 3 or more sons. Of those men joining the study, all but one had either all sons or all daughters, one man had three sons and one daughter.

5.6.2 Clinical semen analysis results

Arrangements were made for each of the subjects recruited to produce a semen sample at Concept Fertility Centre and a semen analysis was performed on all samples received. Following the results of the semen analysis, 3 men did not proceed with the study because their samples proved to be severely oligospermic, with almost 0% motility. The 3 individuals were invited back to provide a second sample, as there was the possibility of incomplete collection. Only 1 of these subjects provided a second sample, and the semen analysis confirmed that it was severely oligospermic.

Table 5.15 summarises the results of the semen analysis for the other 24 donors. A number of interesting findings emerged when the results of the semen analyses from men with three or more sons were compared with those from men with three or more daughters. There were highly significant differences between the volume of the ejaculate, the total count in millions/ml and the motile count in millions/ml. The biological significance of these results is difficult to define, especially when there was no statistically significant difference between the mean total and motile counts of the whole ejaculate. Due to the relatively small size of the sample population, further subjects would have to be sampled to determine whether the results were meaningful.

Table 5.15: Clinical semen analysis results

	Mean volume [SE]	Mean total count (millions/ml) [SE]	Mean motile count (millions/ml) [SE]	Mean total count in ejaculate [SE]	Mean motile count in ejaculate [SE]
All samples	3.40 [0.21]	97.21 [6.34]	68.08 [5.21]	311.74 [23.08]	207.85 [15.20]
Samples from men with more sons	4.08 [0.35]^{A}	73.01 [7.19]^{B}	48.23 [5.40]^{C}	296.94 [37.38]^{D}	193.17 [24.40]^{E}
Samples from men with more daughters	2.72 [0.12]^{A}	121.41 [8.46]^{B}	87.93 [7.34]^{C}	326.54 [27.47]^{D}	222.53 [18.16]^{E}

A: $t = 3.61$, $df = 29$; $p = 0.001$ B: $t = -4.99$, $df = 29$; $p = 0.001$

C: $t = -0.75$, $df = 29$; $p = 0.461$ D: $t = -5.19$, $df = 29$; $p = 0.001$

E: $t = -1.15$, $df = 29$; $p = 0.259$

The samples were next examined to determine whether men who had children of the same sex showed altered percentages of X- and Y- sperm. As can be seen in Table 5.16, there was no significant difference between the percentage of Y- bearing sperm in the semen of men with predominantly sons and those who had predominantly daughters. Where possible, the donors were requested to produce two semen samples, thus allowing the reproducibility of the protocols to be determined eight (35%) of donors provided two samples. When the results obtained with the first and second semen samples were compared, no significant differences were observed in any of semen analysis parameters or the percentage of X- or Y- bearing sperm within the semen.

Table 5.16: Percentage of X- and Y- bearing sperm within the clinical semen samples

	Mean % of Y- bearing sperm [SE]
All samples	52.21 [0.51][A]
Samples from men with more sons	50.79 [0.72][A]
Samples from men with more daughters	53.62 [0.63][A]

A: $t = 1.61$, $df = 20$; $p = 0.123$ (Hybridisation efficiencies of between 90 - 95%)

5.6.3 ISolate® discontinuous gradients

From Tables 5.17 and 5.18 it can be seen that there was no significant enrichment in the percentage of either X- or Y- bearing sperm. There also appeared to be no benefit in terms of enrichment of either X- or Y- bearing sperm by having recoveries of less than 10%. To investigate the variation in the percentage of X- bearing sperm within the samples, the percentage change in X-bearing sperm between the samples was investigated as shown in Table 5.19.

Table 5.17: Summary of recoveries and motilities of ISolate® Clinical samples

Stage	n	Mean recovery (%) [SE]	Mean motility (%) [SE]
8-layer ISolate®	21	14.68 [2.18]	98.17 [0.50]

Table 5.18: Summary of FISH results from ISolate® processed clinical samples

Sample	Mean % X in neat semen [SE]	Mean % X in ISolate® final fraction [SE]	Mean % X in culture [SE]
All samples	50.26 [0.86]	51.04 [0.80]	48.06 [1.32]
Samples with recoveries of <10%	50.83 [1.58]	51.13 [1.28]	48.13 [1.99]
Samples with recoveries of >10%	49.78 [0.92]	50.96 [1.06]	47.99 [1.86]

(Hybridisation efficiencies of betwenn 92 - 97%)

Table 5.19: Summary of percentage change between stages of ISolate® protocol

Sample	Mean % change of X - neat semen to ISolate® final prep. [SE]	Mean % change X - neat semen to culture [SE]	Mean % change of X - ISolate® final prep. to culture [SE]
All samples	0.70 [1.10]^{ACD}	-2.24 [1.68]^{AC}	-2.94 [1.08]^{AD}
Samples with recoveries of <10%	0.30 [1.60]	-2.70 [2.52]	-3.00 [1.90]
Samples with recoveries of >10%	1.03 [1.58]^{BEF}	-1.86 [2.35]^{BE}	-2.88 [1.30]^{BF}

A: df = 2, F = 5.15; p = 0.01 B: df = 2, F = 3.54; p = 0.05 C: t = 2.71, df = 10; p = 0.05

D: t = 2.33, df = 10; p = 0.04 E: t = 2.71, df = 19; p = 0.01 F: t = 2.60, df = 19; p = 0.02

From the FISH results of the ISolate® discontinuous gradient protocol, the only significant findings were obtained with the percentage change between

the three stages of the methodology. Significant differences were observed between all the stages when all the samples were compared ($p < 0.05$), and also when only those samples with greater than 10% recoveries were compared ($p<0.05$). Although a statistically significant difference in the percentage of X-bearing sperm is observed between the three stages of the protocol, the level of enrichment is of no clinical value.

In the samples processed using the ISolate® protocol, there were two pairs of samples from the same donors. When the results from these duplicate specimens were compared, it was found that there was no significant difference between the results, thus suggesting that the results obtained were acceptably reproducible.

5.6.4 Human serum albumin columns

There were no significant differences observed between the FISH results obtained with the HSA neat semen, the HSA 2-layer ISolate® pre-treated preparation and the HSA final fraction (Tables 5.20 and 5.21). There also was no significant difference in the percentage changes between the three groups of samples (Table 5.22).

Samples from the same donor were compared to determine the reproducibility of the techniques and also to see if the results of the semen analysis was constant. There were no statistically significant differences between any of the parameters from the HSA columns.

Table 5.20: Summary of recoveries and motilities of HSA Clinical samples

Stage	n	Mean recovery (%) [SE]	Mean motility (%) [SE]
2-layer ISolate®	9	31.27 [10.68]	66.89 [9.28]
7.5 min HSA column	9	11.36 [3.92]	94.11 [3.20]

Table 5.21: Summary of FISH results from HSA clinical samples

Sample	Mean %X in neat semen [SE]	Mean %X in ISolate® pre-treatment fraction [SE]	Mean %X in HSA final fraction [SE]
All samples	48.38 [1.04]	46.64 [1.62]	49.85 [2.09]
Samples with recoveries <10%	47.44 [1.45]	46.67 [2.58]	48.90 [2.17]
Samples with recoveries >10%	49.93 [1.04]	46.60 [1.48]	51.43 [4.80]

(Hybridisation efficiencies of between 93 - 95%)

Table 5.22: Summary of percentage change between stages of HSA protocol

Sample	Mean % change of X - neat semen to ISolate® pre-treatment prep. [SE]	Mean % change of X- neat semen to HSA final prep. [SE]	Mean % change of X - ISolate® pre-treatment prep. to HSA final prep. [SE]
All samples	-1.74 [0.98]	1.50 [2.15]	3.21 [2.68]
Samples with recoveries <10%	-0.78 [1.37]	1.50 [1.96]	2.24 [2.80]
Samples with recoveries >10%	-3.33 [0.83	1.50 [5.49	4.83 [6.21]

6. Discussion

6.1 Introduction

Couples have many different reasons for wanting to choose the sex of their children. However, whatever the reasons given for wishing to select the sex of a child, there are numerous ethical considerations, serveral of which have already been discussed.

In the past twenty years in excess of a hundred major scientific articles have been published discussing different methods for preselecting the sex of a child. With most of these methods there is no conclusive scientific evidence that they work. Nevertheless, a number of different techniques have been adopted for routine use in Gender Selection Clinics in the United States of America and Europe.

In the following discussion, the results of the present study will be specifically evaluated. It will be seen that the sex preselection methods studied were not successful in terms of X- or Y- bearing sperm enrichment, and The study also failed to demonstrate any significant difference between the ratio of X- and Y-bearing sperm in the semen of men with predominantly sons or daughters. From a technical perspective the investigation did however confirm that dual colour FISH was a suitable method for the routine determination of the percentage of X- and Y- bearing sperm within a fraction of semen or processed sperm.

6.2 Response to the study

In the preparation stages of the study, there was concern over possible difficulties in the recruitment of the subjects required. It was decided that a minimum of six subjects would be needed, however, due to the criteria in place (i.e. three or more children of the same sex) and the potentially delicate nature of the study, no subjects had been recruited prior to the invited assistance given by the Public Relations department of the King Edwards Memorial Hospital.

There was a significant response to the article written on the study in The West Australian, and following its publication further publicity resulted on both local television and radio. Concept Fertility Centre received a number of telephone enquires about the project, with approximately fifty telephone calls logged. Because of the greater than expected response, it was decided that the number of subjects in the study population should be increased. With the agreement of the University Ethics committee, the number of men recruited was increased to include all of those expressing an interest in being included in the study.

From these calls several matters emerged. One of the more concerning findings in ethical terms was the number of people who strongly desired a child of a specific sex and who were prepared to use possibly unreliable methods in the hope that their wishes could be met. Several of the enquiries received were from individuals (all of whom were women) who thought that sex preselection techniques were available from the Centre. Each of these individuals expressed an interest in achieving a pregnancy using selection techniques. It was explained to all callers that the present study was undertaken solely to evaluate the

techniques, and under no circumstance would any attempts to achieve pregnancies be initiated. However three individuals failed or were unwilling to accept this information, believing that the article and the subsequent publicity implied that the techniques were available, and they wished to proceed with these unproven techniques. Once these individuals accepted the fact that there was no possibility of insemination, all three decided against joining the study. Their decisions not to join the study was beneficial because, following discussions with Dr. Matson, it was decided that individuals with such strong emotions about achieving a pregnancy using unproven, and as now determined unsuccessful techniques, would not be appropriate for inclusion in the study.

In addition to the above-mentioned telephone calls, requests to join the study were received from a man who did not have any children and one who had only one daughter. On the basis of the declared aims, it was decided that they were not suitable and therefore their requests were declined.

All of the men who were interested in joining the study were invited to Concept Fertility Centre to discuss the study in greater detail. Initially either Dr. Matson or myself discussed the studies with the potential donors. However three men expressed some concern about discussing the project details with me because of the delicate nature of the subject. Since their personal preference was to speak to a male, it was decided that Dr. Matson should explain the details of the study to persons who were considering inclusion in the study.

Almost all of the men who enlisted in the study expressed a strong desire not only to receive a summary of the study, but also specific information on the semen analysis and processing of the semen samples they provided. For this

reason it was decided that all the subjects recruited should receive the results of their specific samples.

6.3 Semen analyses

At the beginning of the project it was decided that semen analysis should be conducted on each sample, however the morphology of the sperm within the individual samples was not studied. As previously discussed, this decision was taken because of the debate on the influence of sperm morphological characteristics on their fertilising capacity (Dunphy, Neal & Cooke, 1989; Hargreave & Elton, 1983). It was also assumed that all the men in the study had proven fertility, as each was the declared father of at least three children. However, the samples provided by three of the men were severely oligospermic. One possible explanation was that the oligospermia was a manifestation of production, however it was difficult to fully investigate these cases due to the limited time available. The results of the semen analyses carried out on the samples provided by these subjects were discussed with them by Dr Matson.

From the information received in the semen analyses, it was shown that there was a significant difference between the total ejaculate volume and the total and motile counts measured in millions/ml between the samples from men who had three or more sons and those who had three or more daughters. In view of the limited number of samples involved it is difficult to estimate the relevance of these results. However, it seems unlikely that either of these factors will have an influence on determining the sex of a child.

6.4 Ratio of X- and Y-bearing sperm in neat semen

One of the main aims of this study was to determine if men with multiple children of the same sex had an altered ratio of X- or Y- bearing sperm within their semen. Previous studies have stated that the ratio within semen was approximately 1:1 X-: Y- bearing sperm, for example Wang (1994a). Other studies, such as that by Bibbins (1988), did observe an altered ratio of X to Y- bearing sperm in a population consisting of men who had fathered three or more daughters and no sons. In this example, the mean percentage of X- bearing sperm was 68%, however the percentage of Y- bearing sperm in the samples was determined using quinacrine fluorescence staining which, as discussed in chapter 2.6, is now considered unreliable.

In the present study no significant variation from the expected 1:1 ratio of X- to Y- bearing sperm was observed. Nor was there any significant difference between the percentage of Y- bearing sperm within the semen of men with three or more sons compared with that from men with three or more daughters. Therefore it would seem there is no obvious predisposition to having children of a certain sex, however the results are from only one or two semen analyses carried out within a 28-day period. It would be interesting to determine if the percentage of X- or Y- bearing sperm was constant for any man. However, as sperm maturation takes 72 days on average, a study of this nature would necessitate measurement of the percentage of X- and Y- bearing sperm within the semen over a period of at least 12 weeks. The advantage would be to determine if there were any fluctuations through time from the expected 1:1 ratio of X- to Y- bearing

sperm, which in turn might explain why these men might have had a greater than equal chance of having children of only one sex.

Another possibility to be considered is that either X- or Y- bearing sperm may be advantaged during their ascent of the female reproductive system. Alternatively, some form of selection may exist as to which type of sperm is most capable of penetrating the ovum. In fact, it has been suggested that is it possible to select the sex of a child due to variations in the surface charge of the ovum membrane (Stephens, 1997). Further, it is known that some individuals have anti-sperm antibodies, and it has been suggested that Y- bearing sperm have H-Y antibodies on their surfaces (see chapter 2.5). Thus it seems possible that some of these anti-sperm antibodies are in fact anti-Y- bearing sperm antibodies, which could result in sex preselection by rendering the Y- bearing sperm incapable of fertilisation.

6.5 ISolate® Discontinuous Gradients

In using these gradients, the aim was to enrich the percentage of X-bearing sperm within a semen sample. The principles behind this process were based on studies originally conducted using discontinuous gradients formed from Percoll. As previously discussed in chapter 3.3, the method was first described in the early 1980s and, it was claimed, produced samples of sperm which contained up to 94% X- bearing sperm (Kaneko, Oshio, Kobayashi, Mohri & Iizuka, 1984) The mechanisms involved in the production of fractions rich in X- bearing sperm are not fully understood, and hence it is difficult to determine the ideal conditions under which to reproduce the success rates in enrichment indicated by the

authors. Theories on how the Percoll gradient selects for X- bearing sperm include separation due to differences in the differential velocity of X- and Y- bearing sperm, or that X- bearing sperm have a greater nett negative charge which could separate the sperm if there was an interaction between the sperm surfaces and the medium.

Studies comparing ISolate® with Percoll show that the media produce final fractions containing sperm of similar characteristics, for example, percentage motility. It is however possible that there are differences between the media which affect the isolation of X- bearing sperm. The ISolate® discontinuous gradients showed no enrichment in X- bearing sperm following processing with an 8-layer ISolate®. It is possible that this lack of enrichment is due to chemical and/or physical differences between Percoll and ISolate®.

6.6 Survival rates of X- and Y- bearing sperm

It was suggested that X- bearing sperm are longer-lived than Y- bearing sperm (Shettles & Rorvik, 1984), therefore aliquots from the ISolate® processed fractions were cultured as described in chapter 5.3.5. A possible reason for claimed enhanced longevity of X- bearing sperm is that their utilisation of ATP is lower because of their allegedly slower rate of motility (Shettles & Rorvik, 1984). However, this theory is based solely upon circumstantial evidence. A number of different sex preselection techniques are based on the idea that X- bearing sperm survive for longer periods of time, and therefore are more likely to fertilise an ovum if intercourse occurs prior to ovulation (Shettles & Rorvik, 1984). For example, one component of the Shettles method of sex preselection involves the

timing of intercourse in relation to ovulation, with selection for a female conceptus requiring that intercourse should take place at least 48 hours before the expected day of ovulation.

The sperm culture techniques carried out during the study involved examination of sperm motility at 24 hour intervals until the number of motile sperm was less than 50% of the initial motile count. By this stage, it should have been possible to determine whether there was an alteration in the ratio of X- to Y-bearing sperm over time. However, no significant alteration was observed when the ratio of X-: Y- bearing sperm following ISolate® treatment was compared with that after in vitro culture.

A problem encountered with the culture process was the very low counts of motile sperm (approximately 1million/ml or less). This makes accurate determination of the ratio of X- and Y- bearing sperm difficult, as the low counts and high degrees of background interface make FISH less accurate. The possible influence of time in culture on the fertilising ability of the sperm also is unknown. It is assumed that because a percentage of the sperm are still motile after in vitro culture, they are also still capable of fertilisation. This however cannot be proven without using tests such as sperm penetration assays or human zona pellucida binding tests (see Table 3.3), which were beyond the scope of this present investigation.

6.7 Human Serum Albumin Columns

This method of sex preselection is based on the supposed isolation of fractions rich in Y- bearing sperm and it is perhaps the most debated technique

so far proposed. Part of the controversy is due to the fact that the technique is already in use in a number of gender selection clinics in both Europe and the United States. With this in mind, the HSA method of sex preselection was included in the study in order to determine if, in fact, Y- bearing sperm enrichment could be observed.

The actual techniques involved are discussed in detail within the body of scientific literature available, and are summarised in chapter 3.2. The methods adopted were based on the research by Ericsson and colleagues (1973). The initial and pilot studies were used to establish a protocol which produced final fractions containing at least 90% motile sperm, but with a recovery of less than 10% of the initial motile sperm, since it had been suggested that these factors are very important in achieving fractions rich in Y- bearing sperm (Pyrzak, 1994).

The actual protocol is relatively straightforward and therefore potentially suitable for routine clinical use, however no statistically significant enrichment in Y-bearing sperm was observed in any of the samples following processing. It was not possible to produce final fractions that had the desired criteria for all samples. Theré was however no significant difference in the percentage of X- or Y- bearing sperm in those samples with either less than 10% recoveries or those with more than 10% recoveries. Due to the difficulties in establishing a protocol that always produced final fractions with the desired recovery of initial motile and percentage motile sperm, it is possible than there was a problem with the protocol as devised for this study. However, as no enrichment was observed in those samples with the prescribed recoveries and motilities, it seems improbable that this is the case, and in addition a number of similar studies have previously failed

to observe any enrichment in Y- bearing sperm (see Wang, 1994a and Vidal, 1993).

6.8 Fluorescence *in situ* hybridisation

Several studies have used fluorescence *in situ* hybridisation to determine the percentage of X- and Y- bearing sperm in a sample, because it is considered to be more reliable than alternative methods such as quinacrine staining. There is a significant body of published scientific information indicating that FISH is reliable and also very suitable for clinical use in measuring the percentage of X- and Y- bearing sperm within a sample.

FISH has not only been employed to determine the percentage of X- and Y-bearing sperm, but can also be used to identify a number of different chromosomal disorders. A common way of confirming the binding of the FISH probes to the correct chromosomal sections usually involves the use of known standard cells. The probes used in the present study and in routine use within the Cytogenetics Department of KEMH were obtained from commercial sources and so had already been tested to confirm correct, specific binding to the target sites.

Some problems were encountered in establishing a suitable method for the decondensation of sperm heads, but once overcome the process proved to be relatively straightforward. After using FISH during the course of this investigation it is understandable why it has become a popular research and clinical tool. Nevertheless, as the incubation period is at least 18 hours, it is not possible to measure the percentage of X- or Y- bearing sperm within a sample

following processing but prior to insemination. Determination of the percentage of X- or Y-bearing sperm within a sample prior to insemination could serve as a means of ensuring that each sample treated by a sex preselection technique does in fact enrich the required group of sperm.

6.9 Overall Conclusions

The fact that no variation from a 1:1 ratio of X to Y- bearing sperm was observed, within the semen of the study population chosen on the grounds of their apparently biased sex ratio, strongly suggests that under normal physiological and environmental circumstances there is a 50% chance of having a child of either sex.

While the study failed to identify a technique which could be routinely used to enrich X- or Y- bearing sperm, the response evoked by the media coverage confirmed that there is a significant demand for such a technique. Therefore further investigation into the reliability of other techniques which have been suggested would appear warranted, for example, involving the use of flow cytometry. Otherwise, couples who desire to select the sex of a child, as in the case of an X-linked disorder, will be dependent on techniques that are unproven in practice and probably of little value in terms of their intended purpose.

Most of the studies so far completed to alter the ratio of X to Y- bearing sperm have involved small study populations and this is also the case with the present study. It is worth remembering that studies into the secondary sex ratio have shown small study populations can produce unreliable results. For example, in a study based on 301 families, Manning et al (1997) claimed that the

age difference between the parents (age of husband – age of wife) predicted the sex of the first child, with a significant difference between the ages of the parents increasing the likelihood of the first child being a son. A second significant larger study using data on 467,693 births in 12 countries indicated that the ages of the parents had no effect on the sex of the first-born child (Arnold and Rutstein, 1997). A finding of this nature demonstrates the need for large tightly controlled confirmatory studies, even when smaller scale investigations may have resulted in statistically significant findings.

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Appendix

Sex test donors wanted

BY CARMELO AMALFI

MEDICAL researchers need sperm donors to take part in a new study to determine whether parents can choose the sex of their child.

Edith Cowan University masters student Jacquelyn Irving and researchers at the King Edward Memorial Hospital concept fertility centre in Subiaco will examine techniques used to separate semen carrying either the X or Y sex chromosomes.

Six donors, preferably with three or more children of the same sex, will be asked to donate two semen samples each.

Centre scientific director Phillip Matson said some clinics, particularly in the US and Europe, specialised in gender preselection.

They claimed to be able to alter the ratio of X and Y and so shift the probability of having a boy or girl.

Dr Matson said the study involved examining two techniques and would either confirm or refute the claims.

"We really want to take a neutral attitude to see whether these claims can be substantiated because I think people are vulnerable and can be exploited," he said yesterday.

Ms Irving, who is conducting the research as part of her masters program at ECU's school of biomedical and sports science, said two techniques would be tested.

One technique involved separating the slightly heavier X chromosome using centrifugation. Once an egg is fertilised, it is the X-bearing sperm which produces girls and the Y-bearing sperm which produces boys.

Under normal circumstances, semen contained approximately equal numbers of each.

But some families have either more boys or girls and would prefer further children to be of the opposite sex.

She said about 7 per cent of Australian families had three or more children of only one sex.

Dr Matson said with links to the cytogenetics department at King Edward, the study team had the opportunity to evaluate methods of identifying the ratio of X and Y chromsomes without the need for inseminating women.

The results could provide valuable information for patients in the future who wanted to use this treatment to influence the chances of bearing a boy or girl.

Human cells contain 46 chromosomes set out in 23 pairs that contain the genetic information necessary to construct a human. Two of those are the X and Y chromosomes.

At conception, a baby collects 23 chromosomes from its mother and 23 from its father — with girls produced when the sperm adds an X chromosome to one in the egg and boys when sperm adds a Y chromosome. Donors interested in taking part should contact Dr Matson on 9382 2388.

Appendix A

A Systematic Evaluation of Methods to Separate Human X- and Y- Bearing Sperm

Concept Fertility CENTRE

Information Sheet For Donors

Thank you for expressing an interest in the above research study, which is a joint venture between Concept Fertility Centre and The Department of Human Biology of Edith Cowan University. The purpose of this study is to examine systematically the value of techniques designed to separate sperm carrying either the X- or Y- chromosome. Upon fertilisation, it is the X- bearing sperm which result in girls and Y-bearing sperm result in boys.

Under normal circumstances, semen contains approximately equal numbers of X- and Y-bearing sperm and the ratio of boys to girls in the general population is close to one. Some families however have a predominance of either boys or girls and would prefer any further children to be of the opposite sex. Several techniques have been described which are claimed to alter the ratio of X- and Y- bearing sperm and so help shift the probability of having a boy or girl. We shall be examining two of these techniques and actually measuring the proportion of X- and Y- bearing sperm in the final preparation. By so doing, we hope to either confirm or refute the efficacy of the techniques.

The results from this study initially will be of research interest only, but could provide important information for other patients in the future who may consider using this treatment to influence the chance of having a boy or girl.

As part of this study you will be asked to produce a semen sample on up to two separate occasions by masturbation at the Concept Fertility Centre or by intercourse using special non-toxic condoms. It should be noted that these samples will not be used to achieve pregnancies.

At the end of this study if you are interested, you can receive copies of the results.

If you are uncertain about any part of the above procedures or have any general queries concerning this study, please do not hesitate to contact Dr. Matson or Jacquelyn Irving at Concept Fertility Centre,

C/O King Edward
Memorial Hospital

374 Bagot Rd Subiaco
Western Australia 6008

PO Box 966
Subiaco WA 6008

Telephone: (08) 9382 2388
Facsimile: (08) 9381 3603

Email. concept@conceptfert.com.au

A Systemic Evaluation of Methods to Separate Human X- and Y- Bearing Sperm

Concept fertility CENTRE

Consent Form

I have been fully informed about all aspects of the above research study. Any questions I have asked have been answered to my satisfaction.

I understand that this research study will be carried out in a manner conforming to the principles set out by the National Health and Medical Research Council.

I agree that the research data gathered for this study can be published provided that I am not identifiable.

I understand that at any time after I have given my consent I have the right to withdraw from this study.

I, ... have been asked to participate in the above research study, which was explained to me by ..... I understand its aims and how I will be involved and hereby give my written consent.

Name:.....

Signature:.....

Date:.....

Witnessed by :.....

Signature of Witness:.....

Date:.....

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In Vitro Laboratory Pty Ltd ACN 009 [illegible]
As Trustee for the IVF Unit Trust

Appendix E

Chemicals Used

Chemical	Supplier	Catalogue No.
α-DIG-RHO (Anti-digoxigenin rhodamine)	Boehringer Mannheim	1207 750
AV-FITC (Avidin Fluorescein Conjugated)	Pierce	21221
Biotinated Anti-Avidin	Pierce	31850
Bovine Serum Albumin (Fraction V)	Sigma	A3350
Citric Acid, trisodium salt dihydrate	Anala R ® BDH	100813M
DABCO (1,4-Diazabicyclo[2.2.2.]Octane)	Sigma	D2144
DAPI (4',6' Diamidino-2-phenylindole)	Sigma	D9522
Dextran Sulfate	Pharmacia	17-0340-01
DTT (Dithiothreitol)	Sigma	D0623
DXZ1 (Digoxigenin labelled)	Oncor	P5060-DG.5
DYZ1 (Biotin labelled)	Oncor	P5061-B.5
Earle's Balanced Salt Solution (EBSS)	Gibco BRL	14055-057
Ethanol	Anala R ® BDH	10107-7Y
Formamide	Anala R ® BDH	10326-6T
Glacial Acetic Acid	Univar Ajax Chemicals	796
Glycerol	Univar Ajax chemicals	242-500ml
Hepes Buffer	Calbiochem	391338
Human Albumin Plasma (FractionV)	Calbiochem	12666
Human Cot1 DNA (1mg/ml)	Boehringer Mannheim	1581 074
Hybrisol VI	Oncor	S1370-30
Hydrochloric Acid	Sigma	H7020
ISolate®	Irvine Scientific	99264
KH_2PO_4	Anala R ® BDH	15318-4U
Methanol	BDH	10158-6B
Mixed-bed ion exchange resin	Bio-Rad	AG501-X8
Na_2HPO_4	Anala R ® BDH	10249-4C
$NaHCO_3$	Univar Ajax Chemical	475-500G
NaOH	Univar Ajax Chemical	482-500G
Penicillin	CSL	09587501
Potassium Chloride	Sigma	P4504
Sodium Chloride	Sigma	S7653
Sodium Hydroxide	Anala R ® BDH	10252-4X

Sodium Pyruvate	Sigma	P2256
Sterile Water	Baxter Healthcare Pty	AHF7114
Streptomycin	CSL	09470201
Tris Buffer	Sigma	T9650
Tween ® 20	Boehringer Mannheim	1332465
Trypsin	Boehringer Mannheim	109827
Whatman ® Filter Paper #1	Selby	366117

Appendix F

Preparation of media and glassware for use in andrology laboratory

Glassware Preparation

All glassware used in the preparation of media and Pasteur pipettes were wshed and sterilised using standard Concept procedure. Pasteur pipettes were washed for 2 hours in a 2% 7x Detergent (Flow Laboratories, Irvine) solution. This was followed by a 2 hour running rinse and 20 ultrapure water flushes and then a 24 hour soak in ultrapure water. The pipettes were again flushed using ultrapure water and finally soaked in ultrapure water for 2 hours before being dried in a hot air oven.

The glassware used in media preparation was subjected to a 30% acid bath for between 1 and 5 days, and then rinsed under running water for 30 minutes. It was next flushed 20 times with ultrapure water and soaked in ultrapure water for 2 hours. After drying in a hot air oven the glassware was packaged in aluminium foil and sterilised by dry heat at 180°C for 3 hours.

Ultrapure water preparation

The ultrapure water used in the production of media and washing of glassware was produced by the Milli-Q®Plus system (Millipore® Ltd). This system produces highly purified water which has a maximum resistivity of 18.2 Mohm-cm and a maximum conductivity of 0.055μSiemen-cm.

Hepes buffer

Hepes	47.6g
NaOH	3.2g

Dissolve the Hepes in 800ml of ultrapure water and the NaOH in 200ml of ultrapure water. The NaOH solution is slowly added to the Hepes solution to adjust the pH to 7.3. The buffer must then stand for at least 24 hours before being used to make Earle's Flushing medium. Any unused NaOH solution is retained to adjust the pH of the Earle's Flushing medium Concept Fertility Centre, u.d. b).

Earle's Culture Medium

Penicillin	0.06g
Streptomycin	0.05g
$NaHCO_3$	2.1g
Sodium Pyruvate	0.104g
10x Earle's Balanced Salt Solution (EBSS)	100ml
Phenol Red	0.005g

Dissolve the penicillin and strepomycin in 10ml of ultrapure water. Dissolve the $NaHCO_3$ in 100ml of ultrapure water. Dissolve the sodium pyruvate in 10ml of ultrapure water. Dissolve phenol red in 10ml of ultrapure water.

Add approximately 400ml of ultrapure water to the 100ml of EBSS and very slowly add the $NaHCO_3$ whilst stirring throughly to prevent precipitation of the calcium. Add the antibiotic solution and approximately 280ml of ultrapure water until the osmolarity is between 280-285 osmol/L. The medium is then filtered and 200ml aliquots are stored at 4°C, the medium must be used within 14 days. The pyruvatic acid is filtered and stored, it is added just prior to use. Upon addition the osmolarity is expected to increase by approximately 1 osmol/L (Concept Fertility Centre u.d. b).

Earle's Flushing Medium

$NaHCO_3$	10.5g
Penicillin	0.3g
Streptomycin	0.25g
0.4M NaOH	approximately 9ml
EBSS	450ml

Dissolve the penicillin and streptomycin into 50ml of ultrapure water. Dissolve the $NaHCO_3$ in 500ml of ultrapure water. Add 45ml of antibiotics and 225ml $NaHCO_3$ solutions slowly to the EBSS, then mix well. Add 378ml Hepes Buffer while mixing throughly. Check the pH and adjust to pH 7.3 with the stock 0.4M NaOH. Add 3350ml of ultrapure water, then recheck the pH and check that the osmolarity is between 280 and 285 osmol/L. If the osmolarity needs to be adjusted add extra ultrapure water. Filter into 200ml aliquots and store at 4°C for up to 6 weeks (Concept Fertility Centre, u.d. b).

Appendix G

Solutions for Fluorescence *in situ* hybridisation

Antifade

DABCO	0.23g
Tris pH 8.0	200µl
Glycerol	9ml

Add DABCO to glycerol in a 15ml tube and heat to 70°C to dissolve. Add Tris and 800µl of sterile water and place on mixing wheel for 2 hours. Separate into 1ml aliquots and store at -20°C (Cytogenetics Department KEMH, no date).

DAPI Counterstain Working Solution

Dissolve 5µl of the diluted DAPI stock solution into 1ml of antifade solution and mix well (Cytogenetics Department KEMH, no date).

DAPI Stock solution

1mg DAPI

Dissolve in 5ml of sterile water and then make a 1/10 dilution in sterile water (final dilution - 20µg/ml). Store in the dark at -20°C (Cytogenetics Department KEMH, no date).

Deionised Formamide

Formamide (redistilled)	500ml
Mixed-bed ion exchange resin	25g

Place formamide and resin in a beaker on a magnetic stirrer for at least two hours. After this time filter formamide twice through Whatman filter paper and store in aliquots of 50ml at -20°C until needed (Cytogenetics Department KEMH, no date).

Denaturation Mix

Deionised formamide 17.5ml

20 x SSC 2.5ml

Dissolve above in 5ml of sterile double distilled water (Cytogenetics Department KEMH, no date).

Detection Solution 1

(per slide, prepared just before use)

4xSSC/1% BSA 20µl

(DIG RHO 1µl

AV-FITC 1µl

Mix all the above in a microcentrifuge tube and briefly spin in a microcentrifuge.

Detection Solution 2

(per slide, prepared just before use)

4xSSC/1%BSA 20µl

BIOT-(AV 1µl

Mix all the above in a microcentrifuge tube and briefly spin in a microcentrifuge.

Detection Solution 3

(per slide, prepared just before use)

4xSSC/1%BSA 20µl

AV-FITC 1µl

Mix all the above in a microcentrifuge tube and briefly spin in a microcentrifuge.

0.1M DDT/ 1% Trypsin

DDT 0.1543g

Trypsin 0.1g

Dissolve above in 10ml of sterile water and aliquot into 1ml tubes. Store at -20°C (Cytogenetics Department KEMH Manual).

10 x Phosphate Buffered Saline (PBS)

NaCl 87g

Na2HPO4 5.7g

KH2PO4 1.35g

Dissolve the above in 800ml of sterile water. Adjust the pH to 7.3 with HCl and then make the final volume up to 1 litre. 100ml of this 10x solution is diluted into 1 litre to produce the 1x PBS used as a working solution (Cytogenetics Department KEMH, no date).

Probe Mix

(per slide, prepared just before use)

Hybrisol VI 10μl

Cot1 DNA 1μl

DXZ1 0.5μl

DYZ1 0.5μl

Mix all the above in a microcentrifuge tube and briefly spin in a microcentrifuge. Place on a preheated heating block at 75°C for 5 minutes, then quench on ice for at least 1 minute before placing on a preheated 37°C heating block for 30 minutes.

3M Sodium Hydroxide

NaOH pellets 12g

The above was diluted in 100ml of sterile double distilled water.

20x SSC

NaCl 175.3g

Sodium Citrate 88.2g

Dissolve the above in 800ml of sterile water, adjust the pH to 7.0 with 10M HCl and make the final volume up to 1 litre. 10ml of this solution is diluted the produce the 0.1x SSC working solution (Cytogenetics Department KEMH, no date).

4x SSC/ 1% BSA

Bovine Serum Albumin (Fraction V) 1g

20 x SSC 20ml

Dissolve the above in 80ml of sterile water and mix well (Cytogenetics Department KEMH, no date).

2x SSC/ 50% Formamide

Deionised Formamide 250ml

20x SSC 50ml

Mix the above with 200ml of sterile water (Cytogenetics Department KEMH, no date).

4x SSC/ 0.05% TW20

20x SSC 200ml

Tween ® 20 500µl

Mix the above with 800ml of sterile water (Cytogenetics Department KEMH, no date).